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# The Na/K-ATPase Signaling Regulates Natriuresis in Renal Proximal Tubule

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## Abstract

For decades, the Na/K-ATPase has been proposed and recognized as one of the targets for the regulation of renal salt handling. While direct inhibition of the Na/K-ATPase ion transport activity and sodium reabsorption was the focus, the underlying mechanism is not well understood since decreases in basolateral Na/K-ATPase activity alone do not appear sufficient to decrease net sodium reabsorption across the renal tubular epithelium. The newly appreciated signaling function of Na/K-ATPase, which can be regulated by Na/K-ATPase ligands (cardiotonic steroids (CTS)) and reactive oxygen species (ROS), has been widely confirmed and provides a mechanistic framework for natriuresis regulation in renal proximal tubule (RPT). The focus of this review aims to understand, in renal proximal tubule, how the activation of Na/K-ATPase signaling function, either by CTS or ROS, stimulates a coordinated reduction of cell surface Na/K-ATPase and sodium/hydrogen exchanger isoform 3 (NHE3) that leads to ultimately decreases in net transcellular sodium transport/reabsorption.

**Keywords:** cardiotonic steroids, natriuresis, renal proximal tubule, Na/K-ATPase, NHE3, signaling, ROS

## 1. Introduction

Since J.C. Skou's discovery in 1957 [1], the energy-transducing Na/K-ATPase has been extensively studied for its ion-pumping function and, later on, its signaling function. While the signaling function was first demonstrated in cardiac myocyte primary culture, the phenomenon has been confirmed in different cell types and animal models. The roles of Na/K-ATPase signaling in renal proximal tubule (RPT) sodium handling and oxidative modification of the Na/K-ATPase  $\alpha$ 1 subunit in Na/K-ATPase signaling were explored both in vitro and in vivo. The findings may explain certain mechanism(s) related to the Na/K-ATPase signaling-ROS amplification loop and subsequent regulation of salt sensitivity.

The RPT mediates over 60% of the filtered  $\text{Na}^+$  reabsorption [2, 3]. There are two  $\text{Na}^+$  reabsorption pathways in RPTs. One is through the transcellular pathway, mainly through the apical  $\text{Na}^+$  entry mainly via NHE3 (and other apical  $\text{Na}^+$ -coupled transporters like  $\text{Na}^+$ -glucose cotransporters 1 and 2, to a lesser extent) and basolateral  $\text{Na}^+$  extrusion through the Na/K-ATPase [2, 3]. A coordinated and coupled regulation of sodium/hydrogen exchanger isoform 3 (NHE3, SLC9A3) and

the Na/K-ATPase is critical in maintaining intracellular Na<sup>+</sup> homeostasis and extracellular fluid volume. The other one is the paracellular Na<sup>+</sup> reabsorption pathway through a tight junction (TJ), which depends on the transepithelial electrochemical force and tight junction permeability. Claudin-2 forms paracellular channels with other protein that are selective for small cations like Na<sup>+</sup> and K<sup>+</sup>, small anion like Cl<sup>-</sup>, as well as water [4–6]. Interestingly, the Na/K-ATPase signaling function is able to regulate the apical/basolateral polarity of the Na/K-ATPase as well as the tight junctions' components like claudins in distal tubule MDCK cells [7, 8].

The Na/K-ATPase belongs to the P-type ATPase family and consists of two non-covalently linked  $\alpha$ - and  $\beta$ -subunits. Several  $\alpha$ - and  $\beta$ -isoforms, expressed in a tissue-specific manner, have been identified and functionally characterized [9–12]. In RPTs, the  $\gamma$ -subunit ( $\gamma_a$  and  $\gamma_b$ , also known as FXYD2, one of the small type I single-span membrane FXYD protein families) also interacts with the  $\alpha_1$  subunit to regulate the Na/K-ATPase activity [13–15]. There is also a fifth member of the  $\beta$ -subunit family, named  $\beta_m$  coded by an ATP1B4 gene, that is predominantly expressed in skeletal muscle. Interestingly, the  $\beta_m$  is not associated with the  $\alpha_1$  subunit like other  $\beta$ -subunits, but accumulated in the nuclear membrane and associated with transcriptional coregulator Ski-interacting protein, which led to the regulation of TGF- $\beta$ -responsive reporter Smad7 [16]. The  $\alpha_1$  subunit contains multiple structural motifs that interact with soluble, membrane, and structural proteins. Binding to these proteins not only regulates the ion-pumping function of the enzyme, but it also conveys signal-transducing functions to the Na/K-ATPase [17–32]. NHE3 belongs to a family of electroneutral mammalian Na<sup>+</sup>/H<sup>+</sup> exchangers [33–35]. In RPT, NHE3 resides in the apical membrane of S1 and S2 segments, mediating transcellular reabsorption of Na<sup>+</sup> and HCO<sub>3</sub><sup>-</sup> and fluid reabsorption [36, 37]. In the kidney, more than 85% of the filtered NaHCO<sub>3</sub> is reabsorbed in the RPTs, and NHE3 contributes up to ~60% of the total reabsorption of this segment [38]. RPT NHE3 secretes the largest portion of net H<sup>+</sup> to the lumen and interacts with HCO<sub>3</sub><sup>-</sup> to form H<sub>2</sub>O and CO<sub>2</sub> which can freely translocate into RPT cytosol. In cytosol, H<sub>2</sub>O and CO<sub>2</sub> form H<sup>+</sup> and HCO<sub>3</sub><sup>-</sup> through carbonic anhydrase catalyzation. Finally, the newly formed cytosolic H<sup>+</sup> will be secreted to the lumen, and HCO<sub>3</sub><sup>-</sup> will be moved to the blood through the basolateral-resided Na<sup>+</sup>/HCO<sub>3</sub><sup>-</sup> cotransporter (NBCe1-A, SLC4A4). This cycling carbonic anhydrase-controlled CO<sub>2</sub>-HCO<sub>3</sub><sup>-</sup> system links the NHE3-mediated H<sup>+</sup> secretion to HCO<sub>3</sub><sup>-</sup> reabsorption, to achieve an acid-base equilibrium [39, 40]. Moreover, vesicular NHE3 activity also regulates endosomal pH and consequently affects receptor-mediated endocytosis as well as endocytic vesicle fusion [41, 42]. Under normal conditions, the Na/K-ATPase resides at the basolateral surface, providing the driving force for the vectorial transport of Na<sup>+</sup> from the tubular lumen to the vascular compartment, while the NHE3 resides at the apical surface providing a rate-limiting Na<sup>+</sup> entry into cells.

## **2. The concept of endogenous cardiotoxic steroids (CTS) as natriuretic hormones**

CTS (also known as endogenous digitalis-like substances) are specific ligands and inhibitors of the Na/K-ATPase, which include plant-derived glycosides such as digoxin and ouabain and vertebrate-derived aglycones such as bufalin and marinobufagenin (MBG). Although the production and secretion of endogenous CTS are not completely understood, both ouabain and MBG have been identified as endogenous steroid hormones whose production and secretion can be regulated by multiple stimuli including angiotensin II and adrenocorticotrophic hormone (ACTH) [30, 43–48]. Endogenous CTS are present in measurable amounts under

normal physiological conditions and are markedly increased under a number of pathological conditions such as sodium imbalance, chronic renal failure, hyperaldosteronism, hypertension, congestive heart failure, acute plasma volume expansion, and preeclampsia [46, 49–59].

Even though digitalis-like drugs have been used to treat heart failure patients for over 200 years, studies have also revealed many extra-cardiac actions of these compounds, such as in response to salt loading in both animal models and human hypertensive patients [29, 57, 60–62]. In addition, low doses of CTS not only induced hypertension in rats but also caused a significant cardiovascular remodeling independent of their effect on blood pressure (BP) [63–66].

Bricker was the first to propose the existence of “the third factor” (named after the glomerular filtration rate as the first factor and the aldosterone as the second factor), and Dahl proposed the existence of a hormonal natriuretic factor that might cause a sustained increase in BP in salt-sensitive hypertensive rats [67, 68]. Subsequently, Bricker, de Wardener, and others proposed that this hormonal natriuretic factor inhibits the Na/K-ATPase, and Blaustein described how an increase in endogenous Na/K-ATPase inhibitors might cause a vascular contractility change and then a rise in BP [67, 69–72]. In 1980, de Wardener and MacGregor summarized the state of research at the time and proposed an insightful scheme explaining how the Na/K-ATPase inhibitor works as a natriuretic hormone [73]. In essence, it was contended that the Na/K-ATPase inhibitor (endogenous CTS) will rise in response to either a defect in renal Na<sup>+</sup> excretion or high salt intake. This increase, while returning Na<sup>+</sup> balance toward normal by increasing renal Na<sup>+</sup> excretion, also causes hypertension through acting on the vascular Na/K-ATPase. With the advances in the field over the decades, much has been learned. The first unequivocal demonstration of ouabain-like substance in the human plasma was reported decades ago [46]. Blaustein and Hamlyn’s laboratory has demonstrated how increases in endogenous CTS change vascular contractility and its effect on BP [74]. However, the pathophysiological significance of endogenous CTS (e.g., as a natriuretic hormone) has been a subject of debate since it was first proposed until Lingrel’s laboratory reported their gene replacement *in vivo* studies, which unequivocally demonstrated that endogenous CTS play an important role in the regulation of renal Na<sup>+</sup> excretion and BP through the Na/K-ATPase [75–77]. Specifically, Lingrel’s group generated several lines of mice in which the mouse endogenous ouabain-insensitive  $\alpha 1$  subunit is replaced by a mutant that alters the ouabain sensitivity of the Na/K-ATPase. For example, they generated a line of “humanized”  $\alpha 1^{S/S}$  mice where the endogenous ouabain-insensitive  $\alpha 1$  is replaced by an ouabain-sensitive (human like)  $\alpha 1$ -mutant and used these mice to explore the role of endogenous CTS in the regulation of renal function and BP. Should endogenous CTS be important for these regulations, an increased CTS sensitivity in  $\alpha 1^{S/S}$  mice would make these mice more sensitive to conditions that raise circulating CTS. Indeed, when ACTH was administered to raise endogenous CTS, it caused much severe hypertension in  $\alpha 1^{S/S}$  mice than their control littermates. Moreover, expression of the ouabain-sensitive  $\alpha 1$ -mutant significantly increased renal Na<sup>+</sup> excretion, confirming the natriuretic function of endogenous CTS as proposed by the pioneers of the field [67, 68, 70–73]. More evidences indicate that increases in endogenous CTS regulate both renal Na<sup>+</sup> excretion and BP through the Na/K-ATPase [74–76, 78, 79].

### **3. The Na/K-ATPase signaling by specific ligands and ROS in RPTs**

Ouabain-stimulated protein-protein interaction and subsequent Na/K-ATPase signaling function were first demonstrated in rat neonatal myocytes, which were



further confirmed and developed in porcine LLC-PK1 cells (an immobilized RPT cell line) and other cell types. CTS-stimulated Na/K-ATPase signaling has been reviewed everywhere [22, 31, 32, 47, 80–83].

In LLC-PK1 cells, ouabain-stimulated Na/K-ATPase signaling increases ROS generation. Other than ouabain, exogenous H<sub>2</sub>O<sub>2</sub> and glucose oxidase-induced H<sub>2</sub>O<sub>2</sub> also activate Na/K-ATPase signaling pathways including phosphorylation of c-Src and ERK1/2, as well as protein carbonylation modification of Na/K-ATPase (direct carbonylation of two amino acid residues, Pro<sup>222</sup> and Thr<sup>224</sup>, in the actuator domain of the  $\alpha$ 1 subunit) [84–87]. Pretreatment with antioxidant *N*-acetyl-L-cysteine (NAC) or disruption of the Na/K-ATPase/c-Src signaling complex attenuated ouabain- and glucose oxidase-stimulated Na/K-ATPase/c-Src signaling, protein carbonylation, redistribution of Na/K-ATPase, and inhibition of active transepithelial <sup>22</sup>Na<sup>+</sup> transport. A basal level of ROS is critical in initiating ouabain-stimulated Na/K-ATPase/c-Src signaling, and carbonylation modification of the  $\alpha$ 1 subunit is involved in a feed-forward mechanism of the regulation of ouabain-mediated Na/K-ATPase signal function and subsequent Na<sup>+</sup> transport. Furthermore, a stable overexpression of rat  $\alpha$ 1-mutant Pro<sup>224</sup>/Ala (Pro<sup>224</sup> of rat  $\alpha$ 1 is the same as the Pro<sup>222</sup> of pig  $\alpha$ 1) prevented ouabain-stimulated signal function of Na/K-ATPase, protein carbonylation, Na/K-ATPase endocytosis, and ouabain-induced inhibition of active transepithelial <sup>22</sup>Na<sup>+</sup> transport [79, 86, 87]. Taken together, in LLC-PK1 cells, there is a positive-feedback amplification loop of Na/K-ATPase signaling and ROS generation, in which carbonylation of the Pro<sup>222</sup> of the  $\alpha$ 1 subunit is critical. In this working model, both Na/K-ATPase-specific ligands (such as ouabain) and ROS increases (induced by other stimuli like exogenous added glucose oxidase) could activate the Na/K-ATPase signaling, and the Na/K-ATPase/c-Src complex can function as a “receptor” of ROS signaling. This Na/K-ATPase signaling-ROS axis may explain the role of Na/K-ATPase signaling in the development of different pathophysiological conditions, including RPT sodium handling.

## 4. Endocytosis of Na/K-ATPase

Endocytosis is involved in many important cellular functions. Ouabain-induced endocytosis of the Na/K-ATPase was first observed by the laboratories of Cook and Lamb, which demonstrated that [<sup>3</sup>H]-ouabain (bound to the Na/K-ATPase) was translocated from the plasmalemmal membrane surface to intracellular compartments (lysosomes) in HeLa cells, chick embryo heart cells, and Girardi heart cells [88–92].

### 4.1 Dopamine and PTH

One of the best-studied paradigms of hormonal natriuresis is the renal dopamine system [93–96]. Renal dopamine release increases in response to high salt intake or volume expansion. The activation of D1-like dopamine receptors stimulates PLC- $\gamma$  and cAMP-PKA pathways and increases intracellular Ca<sup>2+</sup>. These pathways work in concert and produce the coordinated downregulation of NHE3 and the Na/K-ATPase and consequently natriuresis [93–95, 97, 98]. While Aperia's laboratory first revealed the pathways involved in dopamine-induced regulation of Na/K-ATPase activity [99–101] that is related to endocytosis of the Na/K-ATPase [102], Moe and others have mapped the pathways of NHE3 phosphorylation and trafficking [103–105]. In RPT, dopamine alters sodium handling by inducing Na/K-ATPase and NHE3 endocytosis. In RPT primary culture of Sprague-Dawley

rats, dopamine-induced clathrin-dependent endocytosis of the rat Na/K-ATPase  $\alpha 1$  subunit is triggered by activation of PI3K and subsequently phosphorylation of Ser-18 of rat  $\alpha 1$  subunit [24, 106–109]. The activation of PI3K also stimulated phosphorylation of the Tyr537 of the  $\alpha 1$  subunit that facilitates its binding with adaptor protein-2 (AP-2), providing the inclusion of the Na/K-ATPase into clathrin-coated pits (CCP) [24, 108]. However, Ser-18 is found only in rat  $\alpha 1$  subunit and is not present in pig and dog  $\alpha 1$  subunits [110]. Depending on the type of renal tubular epithelium, dopamine-induced endocytosis of the Na/K-ATPase may be mediated through PKC- or PKA-dependent mechanisms [108, 111–113]. Parathyroid hormone (PTH)-induced inhibition and endocytosis of the Na/K-ATPase were also demonstrated in opossum kidney (OK) cells, which is clathrin-mediated and requires ERK-dependent phosphorylation of Ser-11 of the  $\alpha 1$  subunit [114].

#### **4.2 Ouabain-induced endocytosis of Na/K-ATPase through Na/K-ATPase signaling**

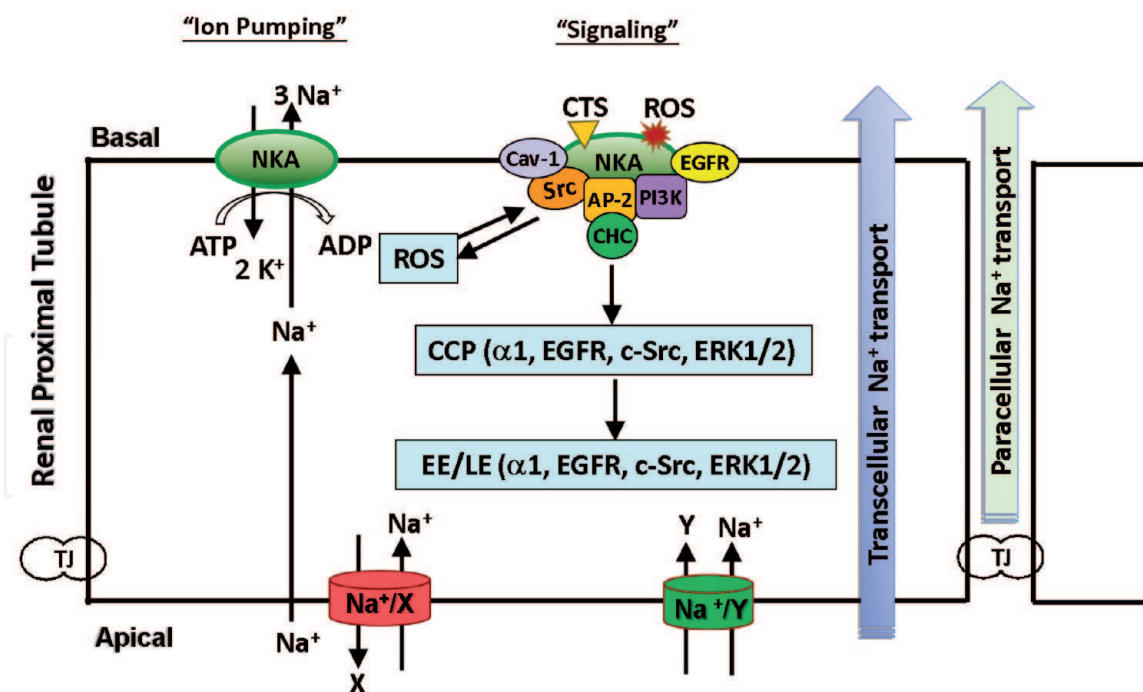
In LLC-PK1 cells, at the doses used, ouabain has no discernable effects on cell morphology, viability, transepithelial electrical resistance, tight junction integrity, and intracellular  $[\text{Na}^+]$  [115]. However, ouabain causes decreases in membrane-bound Na/K-ATPase without significantly affecting intracellular  $[\text{Na}^+]$  [116, 117]. As a specific ligand, nontoxic ouabain (~1/10th–1/20th of acute  $\text{IC}_{50}$ ) caused a dose- and time-dependent decrease in Na/K-ATPase ion-pumping activity (ouabain-sensitive  $^{86}\text{Rb}$  uptake), which is attributed to ouabain-stimulated clathrin-dependent endocytosis of the  $\alpha 1/\beta 1$ -subunits, demonstrated by a decrease in cell surface biotinylated  $\alpha 1$  subunit and a concomitant accumulation of  $\alpha 1/\beta 1$ -subunit and c-Src in early endosome (EE)/late endosome (LE) fractions. This leads to a net decrease in abundance of Na/K-ATPase in the plasma membrane and total ion-pumping activity of Na/K-ATPase and transcellular  $^{22}\text{Na}^+$  transport. This phenomenon was only observed when ouabain was applied to the basolateral, but not apical, aspect of Costar Transwell with membrane support for 12 hours, which indicates that this ouabain-induced endocytosis of the Na/K-ATPase is initiated by activating the receptor Na/K-ATPase/Src complex involving phosphorylation of c-Src and PI3K. The endocytosed [ $^3\text{H}$ ]-ouabain/Na/K-ATPase/c-Src/EGFR complex can be detected in both EE and LE fractions.

To understand the molecular mechanism(s) involved in this process, studies were performed with LLC-PK1 as well as SYF and SYF + c-Src cells. SYF cells are triple Src kinase (c-Src, Yes, Fyn)-null mouse fibroblast cells, and SYF + c-Src are c-Src-rescued SYF cells. This pair of cells was used to determine the role of c-Src activation in ouabain-induced Na/K-ATPase signaling and endocytosis. While ouabain accumulates Na/K-ATPase  $\alpha 1$  subunit content in clathrin-coated pits and EE/LE fractions, it also causes a translocation of the  $\alpha 1$  subunit to nuclear fraction. Interestingly, the effects of ouabain are fully reversible in terms of ion-pumping activity, transepithelial  $^{22}\text{Na}^+$  flux, and cell surface Na/K-ATPase within 24 hours following the removal of ouabain with a fresh culture medium, suggesting a reversible process. Immunofluorescence showed that the Na/K-ATPase  $\alpha 1$  subunit co-localized with clathrin both before and after ouabain treatment, and immunoprecipitation experiments indicated that ouabain stimulated interactions among the  $\alpha 1$  subunit, AP-2, and clathrin heavy chain (CHC). Disruption and/or arresting of clathrin-coated pit formation (by potassium depletion with hypotonic shock [118] and chlorpromazine treatment [119]) significantly attenuated this ouabain-induced endocytosis, suggesting the involvement of a clathrin-coated pit. Inhibition of the ouabain-activated signaling with PP2 (a specific c-Src kinase inhibitor)

or wortmannin (a specific PI3K inhibitor) also significantly attenuated ouabain-induced endocytosis. Experiments performed in SYF cells and SYF + c-Src demonstrated that ouabain induces the endocytosis of the Na/K-ATPase in SYF + c-Src cells, but not in the SYF, indicating that ouabain-induced endocytosis of the Na/K-ATPase is c-Src-dependent.

Ouabain-stimulated Na/K-ATPase signaling also requires caveolin-1 (Cav-1) (a structural protein of caveolae, a subset of membrane lipid rafts) that functions as an anchoring protein for attracting the Na/K-ATPase  $\alpha$ 1 subunit into caveolae [120]. Accordingly, depletion of cholesterol (by methyl- $\beta$ -cyclodextrin (M $\beta$ -CD)) or caveolin-1 (by siRNA) blocked ouabain-induced endocytosis of the Na/K-ATPase, compartmentalization of signaling molecules in clathrin-coated pits, and early endosome. In addition, depletion of caveolin-1 also significantly reduced the protein-protein interactions among  $\alpha$ 1 subunit, AP-2, PI3K, and clathrin heavy chain, suggesting that caveolin-1 is involved in both ouabain-induced endocytosis of Na/K-ATPase and signal transduction [117].

These data demonstrate that ouabain stimulates a clathrin- and caveolin-1-dependent endocytosis of the Na/K-ATPase, a phenomenon requiring ouabain-induced Na/K-ATPase signaling function. Taken together, it is most likely that clathrin- and/or caveola-/lipid raft-mediated endocytosis of the Na/K-ATPase is a common phenomenon, but the mechanism and the relationship between the endocytosis of the Na/K-ATPase and signal transduction are still not fully understood. This is the first time to demonstrate that ligand-modulated endocytosis of the Na/K-ATPase is a mechanism by which RPT sodium transport is altered in a physiologically meaningful manner (Figure 1).



**Figure 1.** Illustration of activation of the Na/K-ATPase signaling-mediated endocytosis of the Na/K-ATPase. Both CTS and ROS can activate Na/K-ATPase signaling, which leads to translocation of cell surface Na/K-ATPase ( $\alpha$ 1- and  $\beta$ 1-subunits), along with EGFR, c-Src, and ERK1/2, into clathrin-coated pits and early and late endosomes. This process is independent of change in intracellular Na<sup>+</sup> and Ca<sup>2+</sup>, but is dependent on activation of c-Src and PI3K, and the presence of caveolin-1. The activation of the Na/K-ATPase signaling also stimulates ROS generation which further activates the signaling. In LLC-PK1 cells, ouabain has no significant effect on recycling of endocytosed  $\alpha$ 1 subunit. AP-2, adaptor protein-2; Cav-1, caveolin-1; CCP, clathrin-coated pits; CHC, clathrin heavy chain; CTS, cardiotonic steroids; EE, early endosome; LE, late endosome; Na<sup>+</sup>/X, Na<sup>+</sup>-dependent antitransporter; Na<sup>+</sup>/Y, Na<sup>+</sup>-dependent cotransporter; NKA, Na/K-ATPase; TJ, tight junction.



## **5. The Na/K-ATPase signaling regulates NHE3 trafficking and activity**

### **5.1 NHE3 regulation**

In RPT, NHE3 resides in the apical membrane of S1 and S2 segments, mediating transcellular reabsorption of  $\text{Na}^+$  and  $\text{HCO}_3^-$  and fluid reabsorption [36, 37]. Moreover, vesicular NHE3 activity regulates endosomal pH and consequently affects receptor-mediated endocytosis as well as endocytic vesicle fusion [41, 42]. Consistent with its cellular function, upregulation of NHE3 activity and expression is associated with the development of hypertension [121–124]. Conversely, the reduction of NHE3 surface expression or NHE3 activity occurs during pressure natriuresis in rats [125–128]. As expected, NHE3-deficient mice are hypotensive [129–131] because of reduced  $\text{Na}^+$  reabsorption and increased  $\text{Na}^+$  excretion. Interestingly, NHE3-deficient mice also develop acidosis since the blunted  $\text{H}^+$  secretion through NHE3, which links to greatly reduced RPT  $\text{HCO}_3^-$  reabsorption (please see Introduction for the linkage of NHE3  $\text{H}^+$  secretion and  $\text{HCO}_3^-$  reabsorption), could not be compensated by  $\text{H}^+$ -ATPase and AE1 (anion exchanger-1, SLC4A1)  $\text{Cl}^-/\text{HCO}_3^-$  exchanger, compared with wild-type mice [131, 132]. These observations put renal  $\text{Na}^+$  reabsorption through NHE3 in a central position in the development and control of salt loading- and volume expansion-mediated hypertension. Structurally, NHE3 has a predicted N-terminal hydrophobic ion-translocating domain and a variable C-terminal hydrophilic domain which contains regulatory sequences [133].

The NHE3 activity is regulated at various levels through different mechanisms, mainly via phosphorylation, trafficking, and transcriptional regulation [34, 35, 103]. The surface expression of NHE3 is mainly regulated by changes in endocytosis/exocytosis and is the primary regulatory mechanism of NHE3 activity. NHE3 has been found to traffic between the plasma membrane and EE/LE fractions via a clathrin- and PI3K-dependent pathway [41, 134–141]. The NHE3 activity can be stimulated by exocytosis [141–143] or inhibited by endocytosis [105, 125, 144]. The activation of c-Src, PKA, and PKC and increase in intracellular  $\text{Ca}^{2+}$  are involved in the regulation of NHE3 trafficking.

NHE3 has been shown to be redistributed under a hypertensive state, accompanying reversible downregulation of the Na/K-ATPase activity in the renal cortex [125, 127, 145]. This raised the possibility that the basolateral-localized Na/K-ATPase and apically localized NHE3 work in concert to regulate renal sodium handling in response to the Na/K-ATPase signaling. The coordinated regulation of NHE3 and the Na/K-ATPase is critical in maintaining intracellular  $\text{Na}^+$  homeostasis and extracellular fluid volume. It is believed that the apical  $\text{Na}^+$  entry through NHE3 is the rate-limiting step because the functional reserve of the Na/K-ATPase in the nephron is more than sufficient even under some pathological conditions.

### **5.2 Chronic NHE3 regulation by Na/K-ATPase signaling**

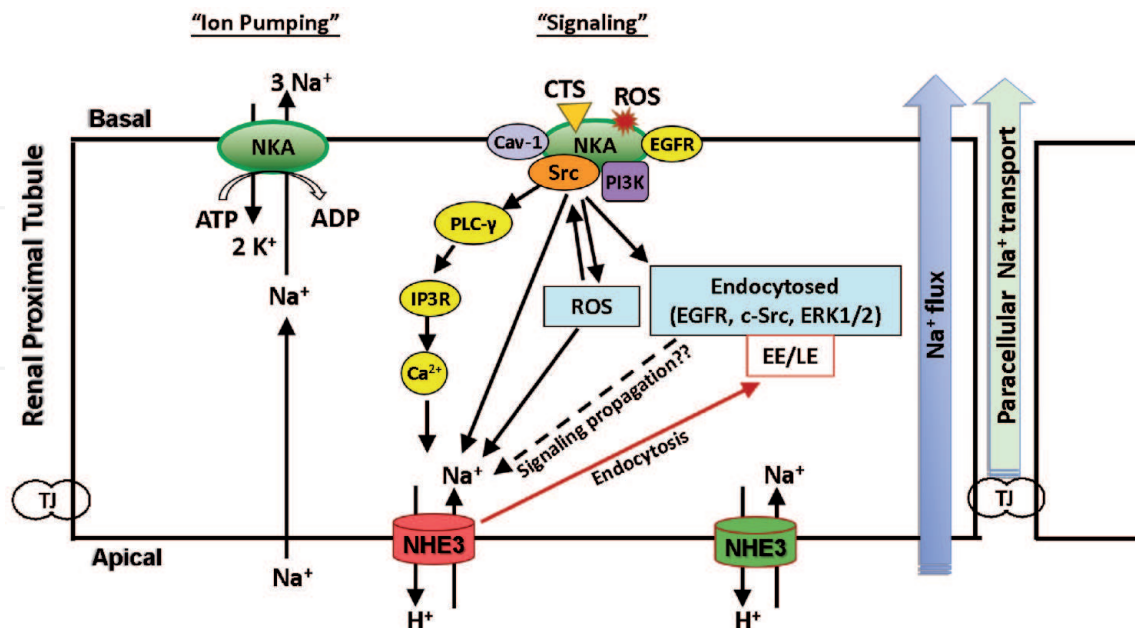
In LLC-PK1 cells, chronic, low-concentration ouabain (50 and 100 nM, 24 hours) treatment in the basolateral aspect, but not in apical aspect, did not change intracellular  $[\text{Na}^+]$  but decreased apical NHE3-mediated  $\text{Na}^+$  absorption, NHE3 promoter activity, and NHE3 protein and mRNA abundance. Pretreatment with specific inhibitors against c-Src and PI3K attenuates ouabain-induced downregulation of NHE3 activity and NHE3 mRNA [146]. In caveolin-1 knockdown LLC-PK1 cells, ouabain failed to reduce NHE3 mRNA and NHE3 promoter activity, in which ouabain-induced Na/K-ATPase signaling reduced Sp1 and TR DNA



binding activity and consequently decreased NHE3 expression and activity [146]. These effects are abolished by inhibition of either c-Src or PI3K. Promoter mapping identified that ouabain-response elements reside in a region between -450 and -1194 nt and that ouabain reduces the binding of transcriptional factor *Sp1* to its cognate *cis*-element.

### 5.3 Acute NHE3 regulation by Na/K-ATPase signaling

Acute application of ouabain (1 hour) in the basolateral, but not apical, aspect significantly reduced NHE3 activity ( $^{22}\text{Na}^+$  uptake) and active transepithelial  $^{22}\text{Na}^+$  transport. This is accompanied by a reduced NHE3 content on cell surface and an increased NHE3 content in EE/LE fractions, as seen in the case of the Na/K-ATPase  $\alpha 1$  subunit. These changes are independent of change in the integrity of tight junctions and the intracellular  $\text{Na}^+$  concentration [115]. Ouabain-induced NHE3 trafficking was abolished by either PI3K or c-Src inhibition. Disruption of caveolae/lipid rafts by cholesterol depletion prevented ouabain-induced accumulation of NHE3 and Na/K-ATPase  $\alpha 1$  in early endosomes, and cholesterol repletion restored the ouabain-induced endosomal accumulation of NHE3 and Na/K-ATPase  $\alpha 1$ . Moreover, pretreatment of cells with the intracellular  $\text{Ca}^{2+}$  chelator BAPTA-AM attenuated ouabain-induced NHE3 trafficking, suggesting  $\text{Ca}^{2+}$  might link the Na/K-ATPase signaling to NHE3 regulation which is in agreement with observations that intracellular  $\text{Ca}^{2+}$  can regulate NHE3 activity and trafficking [147, 148]. These changes indicate that ouabain acutely stimulates NHE3 trafficking, like Na/K-ATPase, by activating the basolateral Na/K-ATPase signaling complex [115]. In RPT cell lines (human HK-2, porcine LLC-PK1, and AAC-19 originated from LLC-PK1 in which the pig  $\alpha 1$  was replaced by ouabain-resistant rat  $\alpha 1$ ), results further indicate that ouabain-induced inhibition of transcellular  $^{22}\text{Na}^+$  transport



**Figure 2.**

*Illustration of activation of the Na/K-ATPase signaling-mediated endocytosis of NHE3. Activation of the Na/K-ATPase signaling leads to intracellular  $\text{Na}^+$ -independent NHE3 endocytosis. However, like Na/K-ATPase signaling-mediated Na/K-ATPase endocytosis, the NHE3 endocytosis is dependent on intracellular  $\text{Ca}^{2+}$ , activation of c-Src and PI3K, and caveolin-1. In LLC-PK1 cells, ouabain inhibits the endocytic recycling of endocytosed NHE3. Since the Na/K-ATPase and NHE3 reside on basolateral and apical membrane in monolayer, respectively, it is still unclear how the basolateral Na/K-ATPase signaling is transmitted to NHE3 regulation. There are several possible pathways as illustrated, as proposed in the text (please see Figure 1 for abbreviations).*

as well as trafficking of the  $\alpha 1$  subunit and NHE3 is not a species-specific phenomenon. Furthermore, in LLC-PK1 cells, ouabain inhibited the endocytic recycling of internalized NHE3, but has no significant effect on recycling of endocytosed  $\alpha 1$  subunit [149].

Taken together, by activating the basolateral receptor Na/K-ATPase/c-Src complex, ouabain can simultaneously and coordinately regulate trafficking of basolateral Na/K-ATPase and apical NHE3, leading to inhibition of transepithelial  $\text{Na}^+$  transport. This mechanism may be important to RPT  $\text{Na}^+$  handling during conditions associated with increases in circulating endogenous CTS. However, it remains to be established whether ouabain-induced regulation of NHE3 trafficking comes from the endocytosed Na/K-ATPase/c-Src complex or directly from the plasma membrane, since ouabain still binds to endocytosed Na/K-ATPase (Figure 2).

## **6. Ouabain-induced regulation of Na/K-ATPase $\alpha 1$ subunit and NHE3 is independent of intracellular $[\text{Na}^+]$**

High concentrations of ouabain are known to increase intracellular  $[\text{Na}^+]$ , depolarize the proximal tubule, and affect the tight junction of epithelial cells. In LLC-PK1 cells, ouabain (up to 100 nM) has no acute effect on intracellular  $[\text{Na}^+]$ , transepithelial electrical resistance, and tight junction integrity, suggesting that in the concentration, ouabain is not likely to increase passive  $\text{Na}^+$  transport by depolarizing LLC-PK1 monolayers [115]. To further define whether the effects of ouabain on the Na/K-ATPase and NHE3 are independent of intracellular  $[\text{Na}^+]$ , the change in intracellular transporters after the equilibrium of intracellular  $[\text{Na}^+]$  with extracellular  $[\text{Na}^+]$  was achieved by using conventional “ $\text{Na}^+$ -clamping” methods [150]. LLC-PK1 cells (both control and ouabain-treated) are pretreated either with 20  $\mu\text{M}$  monensin or with 10  $\mu\text{M}$  monensin plus 5  $\mu\text{M}$  gramicidin for 30 min. Both “clamping” methods raise basal levels of  $\alpha 1$  and NHE3 in EE/LE fractions (monensin is known to accumulate proteins in intracellular compartments). However, ouabain is still able to further accumulate more  $\alpha 1$  and NHE3 in EE/LE. These observations indicate that ouabain-induced trafficking of  $\alpha 1$  and NHE3 can be independent of intracellular  $[\text{Na}^+]$  change [115].

## **7. Coordinated and coupled regulation of Na/K-ATPase and NHE3 by Na/K-ATPase signaling**

Although the mechanisms are still being elucidated, accumulating evidence supports the notion that the expression and activity of the basolateral Na/K-ATPase and apical NHE3 are coordinated and coupled under certain circumstances. For example, McDonough’s laboratory has shown that, during pressure natriuresis and salt loading, the surface expression and activity of both NHE3 and the Na/K-ATPase are simultaneously downregulated to remove  $\text{Na}^+$  from the body [125, 127, 145, 151]. During the development of hypertension in spontaneous hypertensive rat (SHR), the expression and activity of both the Na/K-ATPase and NHE3 are elevated in comparison with the normotensive control rats [121, 152–155].

Activation of Na/K-ATPase signaling, by either ouabain or a high-salt diet, is also capable of stimulating a coordinated and coupled downregulation of apical NHE3 and basolateral Na/K-ATPase to inhibit active transepithelial  $\text{Na}^+$  transport in cultured or isolated RPTs [79, 115–117, 149]. This coordinated regulation depends on activation of the Na/K-ATPase signaling function, but not on acute inhibition

of the Na/K-ATPase activity since it requires the activation of Src and PI3K and increase in intracellular  $\text{Ca}^{2+}$ . Moreover, MBG infusion also induced endocytosis of RPT Na/K-ATPase in rats, which could be prevented by an antibody-mediated neutralization of infused MBG [156].

A high salt intake or volume expansion increases both dopamine and CTS. It has been shown that dopamine-induced regulation of RPT Na/K-ATPase of Dahl S rats was defective because of an apparent decoupling between the binding of dopamine to its  $\text{D}_1$  receptor and activation of GPCRs [157–161]. In response to salt loading, Dahl S rats have a similar diuretic, but much less CTS-related natriuretic response than that seen in Dahl R rats [162]. Both dopamine and CTS can regulate the activity and trafficking of RPT Na/K-ATPase and NHE3. Even though the initiating steps and signaling pathways might be different, they share some signaling steps such as the activation of PLC/PKC and calcium signaling. *It will be of interest to further assess whether there is a crosstalk between CTS- and dopamine-activated signaling pathways in the regulation of renal  $\text{Na}^+$  handling.*

In vivo studies suggest the essential role of CTS in modulating renal sodium excretion and BP with different approaches. First, the administration of some (e.g., ouabain) but not all CTS induces natriuresis [163, 164]. Second, in transgenic mice expressing ouabain-sensitive Na/K-ATPase  $\alpha 1$  subunit, both acute salt load and ouabain infusion augment natriuretic responses, which were prevented by administration of an anti-digoxin antibody fragment [75, 76]. Third, immune neutralization of endogenous CTS prevents CTS-mediated natriuretic and vasoconstrictor effects [55, 59, 78, 80]. Fourth, the administration of the ouabain antagonist, rostafuroxin (also known as PST 2238), prevents not only ouabain-induced Na/K-ATPase signaling but also ouabain-induced increase in BP [64]. Finally, in humans, a high salt intake increases circulating endogenous CTS [57, 80, 165]. An increased CTS excretion is directly linked to an enhanced RPT-mediated fractional  $\text{Na}^+$  excretion, but inversely related to age and to age-dependent increase in salt sensitivity [165].

Although the historical focus has largely been on the direct inhibition of CTS on the Na/K-ATPase ion-pumping activity and sodium reabsorption in RPT as well as vascular tone/contractility, decreases in basolateral Na/K-ATPase activity alone do not appear to be sufficient to reduce net RPT sodium reabsorption since the apical NHE3, but not the Na/K-ATPase, is the rate-limiting step.

In contrast, the newly appreciated signaling function of Na/K-ATPase has been widely confirmed and provides a realistic, mechanistic framework that the renal Na/K-ATPase and its signaling play a key role in regulating renal sodium handling. In porcine RPT LLC-PK1 cells, ouabain activates the Na/K-ATPase signaling pathways and consequently redistributes the basolateral Na/K-ATPase and the apical NHE3 in a coordinated manner; this leads to a symmetrical reduction of cell surface Na/K-ATPase and NHE3 content and ultimately decreased net transcellular sodium transport [86, 87, 115–117]. No significant acute change in intracellular  $\text{Na}^+$  concentration was observed [115], further suggesting the coordination of the downregulation of both apical and basolateral sodium transporters. This Na/K-ATPase signaling-mediated regulation of renal tubular epithelial ion transporters was further confirmed in in vivo studies [79, 156]. It has been shown that endocytosis of signaling molecules could be a way to terminate or propagate the signaling and could further regulate endocytosis itself [166–171]. In this regard, it is possible that ouabain- and ROS-induced endocytosis could be an effective way to terminate Na/K-ATPase signaling-mediated oxidant amplification loop by the degradation of carbonylated Na/K-ATPase, to maintain a certain basal level of ROS and carbonylated protein [172].



## 8. Endocytosis and signaling transduction

The clathrin-dependent endocytosis is the main endocytosis pathway for many membrane proteins in mammalian cells [166, 167, 173–175]. Apart from its endocytic function, the clathrin-coated pits also represent a specialized microdomain, where proteins are assembled into active signaling complexes before internalization of some or all of their components [176]. Some molecules involved in transmembrane signaling, such as  $\beta$ -arrestin, RGS-GAIP (a GTPase-activating protein for G $\alpha$ i heterotrimeric G proteins) [177], GIPC (a PDZ domain-containing protein) [178], and Src family kinases [179], have been localized to clathrin-coated pits, suggesting that the interaction with the components of the pit machinery may facilitate some signaling functions of transmembrane receptors.

Caveolae/lipid rafts play a central role in transcytosis and endocytosis [180–184]. Many signaling molecules and membrane receptors are dynamically associated with caveolae, such as the Src family kinases, Ras, PKC, ERK, insulin receptor, platelet-derived growth factor receptor (PDGFR), EGFR, and some entire signaling modules like PDGFR-Ras-ERK, mainly through their interactions with caveolins [182, 185, 186]. Caveolins stabilize caveolae and modulate signal transduction by attracting signaling molecules to caveolae and regulating their activities [186]. There is also evidence that caveolins modulate endocytosis through their interactions with clathrin [187–190]. Interestingly, both caveolin and clathrin heavy chain are substrates of Src kinase [169, 184].

The Na/K-ATPase  $\alpha$ -subunit, c-Src, and caveolin are present in caveolae isolated by a detergent-free method, in adult rat cardiac myocytes, human embryonic kidney (HEK)-293 cells, and LLC-PK1 cells. In adult rat cardiac myocytes, ouabain not only recruits  $\alpha$ -subunit and c-Src to caveolae but also activates caveolar ERK1/2 [191]. Furthermore, some signaling molecules, such as EGFR and c-Src, are also concentrated in clathrin-coated pits and endosomes in response to ouabain [116], suggesting that both clathrin-coated pits and caveolae are involved in ouabain-mediated Na/K-ATPase signal transduction and endocytosis.

The receptor-mediated endocytosis has been shown not only to attenuate ligand-activated signaling but also to continue the signaling on the endocytic pathway, especially from endosomes [166, 167, 192–194]. While endocytosis is important in the activation and propagation of signaling pathways [168, 195, 196], signal transduction can also regulate endocytosis [169, 197]. Endocytic receptor tyrosine kinase (RTK) receptors could control the magnitude of the original signaling responses (generated at the cell surface) or initiate distinct signaling cascades (qualitatively different from that generated at the cell surface) [170]. In polarized epithelial cells, the distribution of RTK substrates could affect cellular responses [118]. The endosomal signaling appears to be dependent on both the receptor and cell type.

In LLC-PK1 cells, ouabain not only induced compartmentalization of Na/K-ATPase, c-Src, EGF receptor, and ERK in early endosomes but also bound to Na/K-ATPase along the endocytic route [116]. Interestingly, caveolin-1 is also present in early or late endosomes. These facts make it possible that endosomal ouabain-Na/K-ATPase/c-Src might be able to propagate its original signaling or to initiate distinct signaling cascades. This is supported by the findings that ouabain-induced NHE3 regulation is mediated by the activation of the receptor function of Na/K-ATPase. Furthermore, endocytosis is required for ouabain to remove basolateral Na/K-ATPase, which induces a significant inhibition of the pumping activity. Moreover, blockade of Na/K-ATPase signaling/endocytosis appears to be sufficient to abolish ouabain-induced trafficking and transcriptional regulation of NHE3.



Although the mechanisms that involved ouabain-initiated endocytosis of the Na/K-ATPase and NHE3 (and expression) are not fully understood, endocytosis of the Na/K-ATPase may play an important role in renal sodium handling. This is because if ouabain induces a significant depletion of plasmalemmal Na/K-ATPase in proximal tubule type cells (rat proximal tubule primary culture, LLC-PK1) but not in distal tubule type cells (rat distal tubule primary culture, MDCK), it will make physiological “sense” in terms of allowing bulk sodium transport (primarily in the proximal tubule) to be altered and leaving fine-tuning (distal tubule) sodium handling intact.

## 9. ROS and the Na/K-ATPase signaling: the possible link from CTS-stimulated signaling to NHE3 regulation

It is well established that both oxidative stress and high BP are a cause and consequence of each other. The increase in oxidative stress occurs in many forms of experimental models of hypertension, including Dahl salt-sensitive hypertension [198–204]. Increases in ROS can regulate physiological processes including renal tubular ion transport, fluid reabsorption, and sodium excretion [79, 205–210]. In particular, increases in ROS regulate the activity and cellular distribution of the basolateral Na/K-ATPase as well as the apical NHE3 and sodium/glucose cotransporter, at least under normal circumstances [79, 151, 208, 211–216]. Oxidative modification can affect the Na/K-ATPase activity through different mechanisms. For example, S-glutathionylation cysteine residue(s) of the Na/K-ATPase  $\alpha$ -subunit can block the intracellular ATP-binding site [217], and S-glutathionylation of cysteine of the Na/K-ATPase  $\beta$ 1-subunit can affect the Na/K-ATPase conformational poise [218, 219]. Oxidant and oxidative modification of the Na/K-ATPase can lead to degradation, functional changes, and formation of Na/K-ATPase oligomeric structure [74, 84–87, 217, 219–230]. In LLC-PK1 cells, increase in ROS generation, induced by either ouabain or glucose oxidase, is critical in the activation of Na/K-ATPase signaling which mediates trafficking of the Na/K-ATPase and NHE3 and transcellular  $\text{Na}^+$  transport [86, 87]. Pretreatment with higher doses, but not a low dose, of NAC attenuated the effect of ouabain on c-Src activation and transcellular  $^{22}\text{Na}^+$  flux, suggesting a role of basal physiological redox status in the initiation of ouabain-induced Na/K-ATPase signaling. While CTS stimulates ROS generation and Na/K-ATPase signaling in different in vitro and in vivo models [63, 85, 231–233], an increase in ROS alone (without the presence of ouabain) by extracellularly added glucose oxidase is also able to activate Na/K-ATPase signaling, indicating that activation of Na/K-ATPase signaling can be achieved by general stimuli like ROS, other than its specific ligands. Glucose oxidase-induced  $\text{H}_2\text{O}_2$  alone also stimulates Na/K-ATPase endocytosis and inhibits active transcellular  $^{22}\text{Na}^+$  transport [85, 86]. The phenomenon of redox sensitivity of the Na/K-ATPase has been demonstrated in different cell types, tissues, and animal species.

In LLC-PK1 cells, both ouabain and glucose oxidase-induced  $\text{H}_2\text{O}_2$  stimulate Na/K-ATPase signaling as well as direct protein carbonylation of Pro<sup>222</sup> and Thr<sup>224</sup> residues of the Na/K-ATPase  $\alpha$ 1 subunit ( $\alpha$ 1-carbonylation) [86]. The Pro<sup>222</sup> and Thr<sup>224</sup> are located in peptide <sup>211</sup>VDNSSLTGESEPQTR<sup>225</sup> [UniProtKB/Swiss-Prot No P05024 (AT1A1\_PIG)]. While the  $\alpha$ 1 subunit is highly conserved among humans, pigs, rats, and mice (the homology is over 98.5%), the identified peptide is 100% identical among these four species. This peptide is located in the actuator (A) domain of  $\alpha$ 1 subunit, and Pro<sup>222</sup>/Thr<sup>224</sup> are highly exposed and facing the nucleotide binding (N) domain of the  $\alpha$ 1 subunit. Upon ouabain binding, Na/K-ATPase undergoes conformational changes, in which the A domain is rotated to the N

domain favoring an E2-P conformation. The structure-function analysis indicates that these conformational changes may affect binding of the  $\alpha 1$  subunit to signaling molecules such as c-Src and PI3K [234]. In addition, the peptide also contains the TGES motif that is the anchor of A domain rotation [234].

Biologically, ROS can oxidize various types of biological molecules including proteins, leading to their functional changes. Through Fenton's reaction,  $H_2O_2$  is reduced to  $HO^\bullet$  by coupling oxidation of reduced ferrous ion ( $Fe^{2+}$ ) to ferric ion ( $Fe^{3+}$ ). This metal-catalyzed oxidation (MCO) process oxidizes proteins by introducing carbonyl groups (such as aldehydes, ketones, or lactams) into the side chains of certain amino acids (such as proline, arginine, lysine, and threonine) that named direct (primary) carbonylation that have been implied in various conditions like chronic renal failure [235–240]. Since Fenton's reaction involves the conversion of  $H_2O_2$  to  $HO^\bullet$ , any specie of ROS with  $H_2O_2$  as an intermediate and/or end product may stimulate the reaction.

Protein carbonylation is reversible (decarbonylation) and may function as a regulatory mechanism of cell signaling [241–244]. We also observed an undefined decarbonylation mechanism, which apparently reverses the carbonylation of the Na/K-ATPase  $\alpha 1$  subunit induced by ouabain [86]. The removal of ouabain from the culture medium reverses ouabain-mediated carbonylation, as seen in the reversed Na/K-ATPase ion-pumping activity [116]. Moreover, inhibition of de novo protein synthesis as well as degradation pathway through lysosome and proteasome does not affect this decarbonylation, which is still poorly understood. It is possible that carbonylation modification might stabilize the Na/K-ATPase in a certain conformational status favoring ouabain binding to the Na/K-ATPase  $\alpha 1$  subunit and ouabain-Na/K-ATPase signaling. Nevertheless, the underlying mechanism might be physiologically significant since the carbonylation/decarbonylation process could be an important regulator of the RPT Na/K-ATPase signaling and sodium handling.

It is reasonable to propose that carbonylation modification of RPT Na/K-ATPase  $\alpha 1$  subunit has biphasic effects. On one hand, physiological and controllable  $\alpha 1$ -carbonylation stimulates Na/K-ATPase signaling and sodium excretion, rendering salt resistance, whereas on the other hand, prolonged exposure to oxidant stress leads to overstimulated  $\alpha 1$ -carbonylation and desensitized Na/K-ATPase signaling, increasing salt sensitivity. First, Dahl S rats show considerably higher basal levels of oxidative stress than R rats, and high-salt diets increase renal oxidative stresses that contribute to salt-sensitive hypertension [202–204]. Second, while high-salt diets increase circulating CTS, a high-salt diet (HS, 2% NaCl for 7 days) stimulates the Na/K-ATPase signaling in isolated RPT from Dahl salt-resistant (R) but not salt-sensitive (S) rats (i.e., impaired Na/K-ATPase signaling in S rats) [79]. Third, CTS- and  $H_2O_2$ -mediated redox-sensitive Na/K-ATPase signaling and  $\alpha 1$ -carbonylation are involved in this signaling process, in a feed-forward mechanism [86]. Fourth, high but not low concentration of NAC is able to prevent  $\alpha 1$ -carbonylation and Na/K-ATPase signaling [86]. Even though it is still not clear of the carbonylation/decarbonylation process, this could be another new regulatory mechanism of Na/K-ATPase signaling. It is reasonable to postulate that prolonged excessive  $\alpha 1$ -carbonylation (by CTS and/or other factors) might overcome the decarbonylation capacity, leading to the desensitization or termination of the Na/K-ATPase signaling function. This is reminiscent of the observations in clinical trials using antioxidant supplements. The beneficial effect of antioxidant supplements is controversial and not seen in most clinical trials with administration of antioxidant supplements [200, 245]. Low doses of antioxidant supplementation may be ineffective, but high doses may be even dangerous since excess antioxidants might become prooxidants if they cannot promptly be reduced in the antioxidant chain [246]. It appears that the balance of the redox status, within a physiological range, may be critical in order to maintain beneficial ROS signaling.

## 10. Endocytosis of Na/K-ATPase and NHE3 in salt sensitivity

In male Sprague-Dawley rats, compared to a normal salt (0.4% NaCl, 7 days) diet, a high-salt (4% NaCl, 7 days) diet increased urinary sodium and MBG excretion. In isolated proximal tubules, a high-salt diet inhibits the Na/K-ATPase ion-exchange activity and enzymatic activity, which is accompanied by a decreased Na/K-ATPase  $\alpha$ 1 content in heavy membrane fraction and an increased Na/K-ATPase  $\alpha$ 1 content in both early and late endosomes. These high-salt diet-mediated changes were ameliorated by administration of an antibody against MBG [156]. Results indicate that a high-salt diet increased MBG production, activated RPT Na/K-ATPase signaling, and induced endocytosis of Na/K-ATPase.

The Dahl R and S rat strains were developed from Sprague-Dawley rats by selective breeding, depending on the resistance or susceptibility to the hypertensive effects of high dietary sodium [247]. In these two strains, the RPT sodium handling is an essential determinant of their different BP responses [248–251]. At the cost of elevated systolic BP, Dahl S rats get rid of excess sodium primarily via pressure natriuresis. In contrast, Dahl R rats get rid of excess sodium primarily via a significant reduction of renal sodium reabsorption without increasing the BP. *In vivo* study indicates that impaired RPT Na/K-ATPase signaling appears to be causative of experimental Dahl salt sensitivity [79]. *In vivo* studies with Dahl R and S rats (Jr strains) demonstrated that impairment of RPT Na/K-ATPase signaling is a causative factor of experimental Dahl salt sensitivity [79]. In Dahl R but not S rats, a high-salt (2% NaCl, 1 week) diet activated RPT Na/K-ATPase signaling and stimulated coordinated redistribution of the Na/K-ATPase and NHE3, leading to increased total and fractional urinary sodium excretion as well as normal BP. However, there are still questions about the underlying mechanism(s) that need to be further investigated, such as the difference of Na/K-ATPase signaling function between Dahl R and S rats, as well as the translation of Na/K-ATPase signaling to NHE3 regulation. Furthermore, low concentration of ouabain causes hypertrophic response both in the heart and kidney, by concentrating the Na/K-ATPase, Src, EGFR, and MAPKs within rat caveolae, and activates the Na/K-ATPase/Src/MAPK signaling pathway [64]. However, there is no simple explanation for this occurrence. First, the  $\alpha$ 1 subunit is essentially the only  $\alpha$  isoform expressed in RPT, and genes coding  $\alpha$ 1 subunit and NHE3 (in rat chromosomes 1 and 2, respectively) are not located in identified and/or proposed BP quantitative trait loci [252]. Second, there is no difference in  $\alpha$ 1 gene (*Atp1a1*) coding [251],  $\alpha$ 1 ouabain sensitivity [253], and  $\alpha$ 1 expression [79] between these two strains. Third, acute salt loading increases circulating CTS (ouabain and MBG) in both S and R rats [162]. These observations suggest that there must be resistance to CTS signaling in the Dahl S rat, a phenomenon that we only partially understand. As discussed above, the carbonylation/decarbonylation process could be another new regulatory mechanism of Na/K-ATPase signaling. It is reasonable to postulate that prolonged excessive  $\alpha$ 1-carbonylation in Dahl salt-sensitive rats might overcome the decarbonylation capacity, leading to desensitization or termination of the Na/K-ATPase signaling function.

## 11. Perspective

As pointed out by Guyton many years ago [254], the kidney is the most important organ in the regulation of Na<sup>+</sup> handling and BP. Dietary salt intake *vs.* renal sodium handling is a key determinant of long-term BP regulation and plays an important role in the pathogenesis of hypertension, with more pronounced effects seen in salt-sensitive patients. Consequently, modest restriction of dietary salt and



diuretic therapy are often recommended for the treatment of resistant hypertension, particularly with the salt-sensitive subgroup [254–258].

Although the relationships among CTS, renal Na<sup>+</sup> handling, and hypertension were proposed many years ago, there has been an explosion of reports supporting this idea. As discussed, reports from Lingrel's laboratory clearly demonstrated a specific role of the isoforms of the Na/K-ATPase and its interaction with endogenous CTS in the regulation of Na<sup>+</sup> excretion and BP in intact animals [75–77]. From the ligand perspective, studies have demonstrated that CTS are present in measurable amounts under normal physiological conditions and that several disease states are associated with elevations in the circulating levels of CTS. The new concept that the Na/K-ATPase has an ion-pumping-independent receptor function (induced by both CTS and ROS) that can confer the agonist-like effects of CTS on intracellular signal transduction is a new mechanism for RPT sodium handling. Moreover, this newly discovered signaling mechanism operates in intact animals in response to CTS stimulation. The Na/K-ATPase has recently emerged as a therapeutic target [259, 260]. A clearer understanding of the mechanisms, in which a CTS-ROS-Na/K-ATPase signaling axis counterbalancing salt retention, would not only have major pathophysiological and therapeutic implications, but also further explain the progressive impairment of renal sodium handling under excessive oxidative stresses such as hypertension, aging, obesity, and diabetes.

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## **Abbreviations**

BP	blood pressure
CTS	cardiotonic steroids
NHE3	sodium/hydrogen exchanger isoform 3
ROS	reactive oxygen species
RPT	renal proximal tubule



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