

Luminal Na^+/H^+ exchange in the proximal tubule

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Abstract The proximal tubule is critical for whole-organism volume and acid–base homeostasis by reabsorbing filtered water, NaCl, bicarbonate, and citrate, as well as by excreting acid in the form of hydrogen and ammonium ions and producing new bicarbonate in the process. Filtered organic solutes such as amino acids, oligopeptides, and proteins are also retrieved by the proximal tubule. Luminal membrane Na^+/H^+ exchangers either directly mediate or indirectly contribute to each of these processes. Na^+/H^+ exchangers are a family of secondary active transporters with diverse tissue and subcellular distributions. Two isoforms, NHE3 and NHE8, are expressed at the luminal membrane of the proximal tubule. NHE3 is the prevalent isoform in adults, is the most extensively studied, and is tightly regulated by a large number of agonists and physiological conditions acting via partially defined molecular mechanisms. Comparatively little is known about NHE8, which is highly expressed at the lumen of the neonatal proximal tubule and is mostly intracellular in adults. This article discusses the physiology of proximal

Na^+/H^+ exchange, the multiple mechanisms of NHE3 regulation, and the reciprocal relationship between NHE3 and NHE8 at the lumen of the proximal tubule.

Keywords Acid-based balance · pH regulation · Volume regulation · Sodium transport · Bicarbonate transport · Chloride transport · Sodium–hydrogen exchange · Sodium–proton exchange

Na^+/H^+ exchange in biology

Na^+/H^+ exchangers (NHEs) are universally present in prokaryotes, lower eukaryotes, and higher eukaryotes, including fungi, plants, and animals [1]. In prokaryotes, fungi and plants, the transmembrane proton electrochemical gradient ($\Delta\mu\text{H}^+$) energizes the extrusion of Na^+ from the cytoplasm. Plasma membrane NHEs characterized to date in animal cells utilize the inward Na^+ gradient created by the activity of Na^+/K^+ -adenosine triphosphatase (ATPase) to extrude H^+ against its electrochemical gradient in an electroneutral fashion.

All NHEs are members of the very large superfamily of monovalent cation–proton antiporters (CPA), which is divided phylogenetically in the CPA1, CPA2, and NaT-DC transporter families [1, 2]. Nine NHE isoforms (NHE1–9) belonging to the CPA1 family and having different tissue and subcellular distribution (Table 1) have been described to date in the human genome [1, 3, 4]. In addition, the human genome contains a sperm-specific NHE (a member of the NaT-DC family) with demonstrated NHE activity [1, 5, 6], as well as two genes termed NHA1 and NHA2 (members of the CPA2 family) which are more closely related to prokaryotic Na^+/H^+ exchangers [1] (Table 1). The expression and function of NHA2 have been recently confirmed [7, 8]. Brett et al. [1] presented an extensive phylogenetic classification of NHEs using 118 eukaryotic NHE genes of the CPA1 family. Based

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Table 1 Mammalian Na⁺/H⁺ exchangers

| Protein | Tissue expression | Cellular distribution | Renal distribution |
|-----------|--|--|--|
| NHE1 | Ubiquitous | Plasma membrane; basolateral membrane of epithelial cells | Basolateral; throughout the nephron, with the exception of the macula densa and intercalated cells of the cortical collecting duct |
| NHE2 | Gastrointestinal tract, skeletal muscle; less in kidney, brain, testis, uterus | Plasma membrane; apical membrane of epithelial cells | Apical; thick ascending limb and distal convoluted tubule |
| NHE3 | Gastrointestinal tract, kidney, gall bladder, epididymis; much less in brain | Plasma membrane; apical membrane and recycling endosomes of epithelial cells | Apical; proximal tubule and thick ascending limb |
| NHE4 | Stomach; less in kidney and brain | Plasma membrane; basolateral membrane of epithelial cells | Basolateral; throughout the nephron; highest expression in thick ascending limb |
| NHE5 | Brain | Plasma membrane and recycling endosomes (synaptic vesicles) | Very-low-level transcript |
| NHE6 | Ubiquitous | Intracellular (recycling endosomes) | Transcript present |
| NHE7 | Ubiquitous | Intracellular (trans-Golgi network) | Transcript present |
| NHE8 | Ubiquitous (intracellular); luminal surface of renal and intestinal epithelia | Intracellular (trans-Golgi network); apical membrane and recycling endosomes of epithelial cells | Apical; proximal tubule |
| NHE9 | Ubiquitous | Intracellular (recycling endosomes) | Transcript present |
| Sperm-NHE | Spermatozoa | Sperm flagellum | Absent |
| NHA1 | Testis (?) | Unknown | Absent |
| NHA2 | Ubiquitous | Mitochondria, endosomes, plasma membrane | Distal convoluted tubule |

on sequence, cellular location, ion selectivity, and inhibitor specificity, NHEs can be divided into two major subfamilies: *intracellular* and *plasma membrane*. The *intracellular* NHE subfamily can be further divided into three clades: (1) the endosomal/*trans*-Golgi clade, which includes one of the oldest eukaryotic NHE genes, as well as human NHE6, NHE7, and NHE9; (2) the NHE8-like clade which includes eight animal NHE genes and interestingly shows similarity to *Drosophila* NHE; (3) the plant vacuolar clade which includes 32 plant NHE genes and the NHE gene of slime mold. The *plasma membrane* NHE subfamily can be divided into two clades: (1) the *recycling clade* which includes only animal genes (24 in total), including human NHE3 and NHE5; (2) the *resident clade* which is restricted to vertebrate NHE genes (25 in total), including human NHE1, NHE2, and NHE4. This classification is extremely useful for understanding differences and similarities in NHE localization, function, and regulation and for identifying the most appropriate models for the study of individual NHEs.

Two members of the NHE family will be discussed here in the context of the renal proximal tubule: NHE3 and NHE8.

Luminal Na⁺/H⁺ exchange in proximal tubule physiology

The existence of Na⁺/H⁺ exchange activity in the mammalian kidney was first inferred in the 1940s by Pitts and

coworkers [9, 10] in a series of studies that, in the words of Gerhard Giebisch, “provided the ground on which modern renal acid-base physiology is based” [11]. Na⁺/H⁺ exchange activity was definitively demonstrated three decades later by Murer et al. [12], followed by Kinsella and Aronson [13]. Another decade passed before the first mammalian Na⁺/H⁺ exchanger, NHE1, was cloned by Sardet et al. [14]. In the early 1990s, several independent groups identified NHE3 as the main proximal tubular NHE isoform [15–18].

To date, two NHEs have been described at the luminal membrane of proximal tubule cells: NHE3 is predominant in adults [17–19], and NHE8 is highly expressed in neonates [20–22]. A developmental switch between the two transporters has been proposed as part of postnatal renal maturation [22, 23] and will be discussed later in this article. NHE1 and to a lesser extent NHE4 are present at the basolateral membrane [24, 25], but their specific roles in proximal tubule function are not known. Most of what we know today about the molecular physiology and regulation of proximal Na⁺/H⁺ exchange pertains to NHE3, which is by far the best-studied luminal isoform.

Luminal membrane Na⁺/H⁺ exchange in the mammalian proximal tubule mediates the isotonic reabsorption of approximately two thirds of the filtered NaCl and water, the reabsorption of bicarbonate, and the secretion of ammonium (NH₄⁺) and contributes to the reabsorption of filtered citrate, amino acids, oligopeptides, and proteins. The mechanisms are summarized in Figs. 1 and 2. The

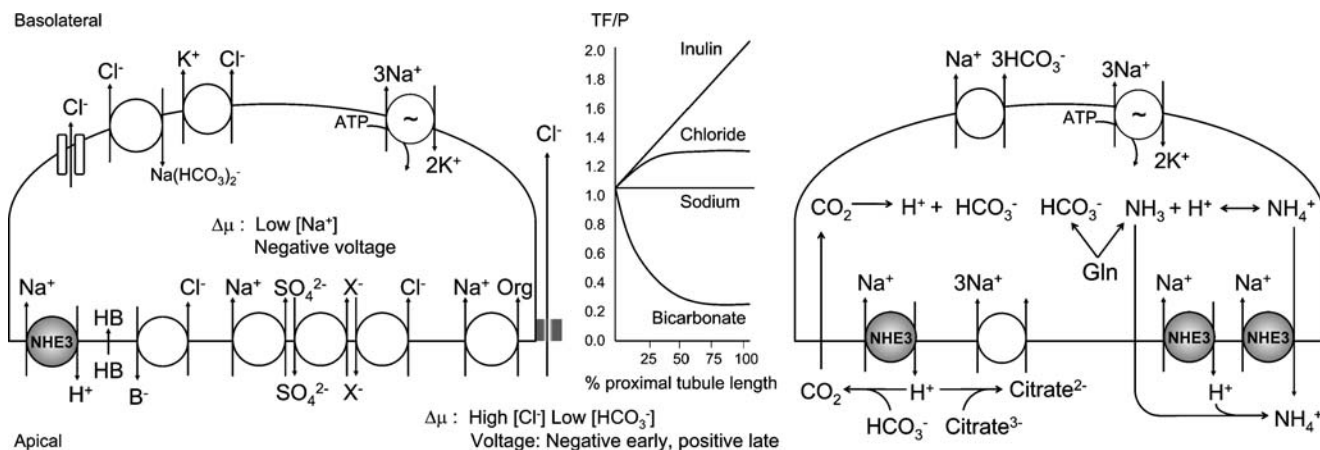


Fig. 1 Model of how apical Na^+/H^+ exchange mediates NaCl absorption (*left*) and luminal acidification (*right*). The electrochemical driving force ($\Delta\mu$) is the low cell $[\text{Na}^+]$ and negative interior voltage generated by the Na^+/K^+ -ATPase. The apical Na^+/H^+ exchanger NHE3 is highlighted in gray. Coupling of Na^+/H^+ exchange with Cl^-/base (B^-) exchange and acid (HB) recycling or triple coupling of Na^+ -sulfate cotransport, sulfate-anion (X^-) exchange and Cl^-/anion exchange constitutes net apical NaCl entry. The Na^+ that enters with organic solutes (Org) also results in net NaCl entry when coupled to paracellular Cl^- transport. Basolateral transcellular Cl^- exit is achieved via diverse mechanisms (Cl^- channel, Na^+ -dependent $\text{Cl}^-/\text{HCO}_3^-$ exchange, and KCl cotransport). Luminal acidification by NHE3

titrates filtered HCO_3^- which results in HCO_3^- absorption. As luminal $[\text{HCO}_3^-]$ falls with isotonic fluid absorption, luminal Cl^- is concentrated (graph in middle panel) along the length of the proximal tubule, which enhances the $\Delta\mu$ for paracellular Cl^- diffusion (TF-P, tubular fluid to plasma concentration ratio). The H^+ extruded by NHE3 also titrate filtered trivalent citrate to bivalent citrate, which is taken up by the Na^+ -citrate cotransporter. Citrate reabsorption is tantamount to base equivalent absorption. The metabolism of neutral glutamine (Gln) generates NH_4^+ (acid) and HCO_3^- (base). NH_4^+ secretion is mediated by NHE3 either by luminal trapping of diffused NH_3 or by NH_4^+ traversing as a substrate. Base exit is mediated by the $\text{Na}^+/\text{HCO}_3^-$ cotransporter

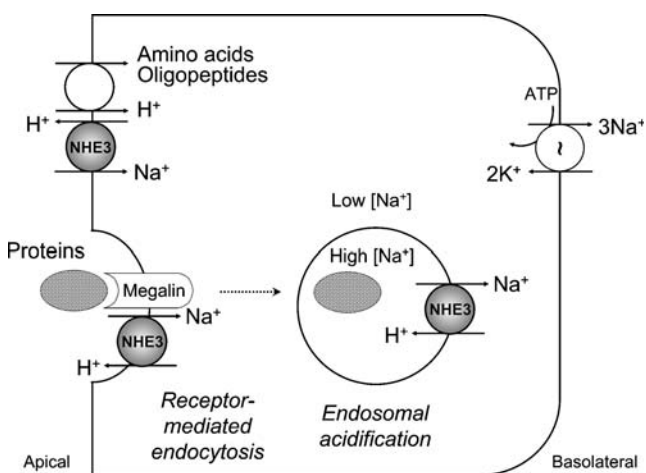


Fig. 2 Model of how apical Na^+/H^+ exchange participates in the proximal tubule reabsorption of amino acids, oligopeptides, and proteins. Apical NHE3 provides the H^+ used for H^+ -coupled cotransport of filtered amino acids and oligopeptides. Apical NHE3 also interacts with megalin, which is responsible for receptor-mediated endocytosis of some proteins in the proximal tubule. The interaction with megalin may recruit NHE3 to endocytic vesicles. After endocytosis, NHE3 (and possibly other intracellular NHEs, not shown) utilizes the outward transvesicular Na^+ gradient of endocytic vesicles and early endosomes to drive inward movement of protons and endosomal acidification, which is important for further processing of reabsorbed proteins. This model requires further experimental validation

regulation of proximal tubule Na^+/H^+ exchange bears relevance to the regulation of these physiological functions.

Net apical reabsorption of NaCl involves coupling of Na^+/H^+ exchange with Cl^-/base exchange and acid recycling or triple coupling of Na^+ -sulfate cotransport, sulfate-anion exchange, and Cl^-/anion exchange [26–28] (Fig. 1, left). In addition, the transcellular cotransport of Na^+ with organic solutes coupled to paracellular Cl^- transport results in net transepithelial NaCl reabsorption [29, 30]. The reabsorption of water results principally from the small osmotic gradient created across the proximal tubular epithelium by NaCl reabsorption. Although water flux can theoretically occur through transcellular and/or paracellular routes, the surface area of the luminal membrane far exceeds that of the paracellular junctions and is endowed with aquaporin water channels [31, 32]. Water is thus mostly reabsorbed via the proximal tubule cell [33, 34].

Proximal tubule bicarbonate reabsorption is in part mediated by apical Na^+/H^+ exchange, which provides the H^+ to titrate luminal filtered HCO_3^- [35–37] (Fig. 1, right). The catalytic action of luminal carbonic anhydrase results in generation of CO_2 , which traverses the apical cell membrane by free diffusion and possibly via water channels, and is reverted to H^+ and HCO_3^- by intracellular carbonic anhydrase [37, 38]. The resulting intracellular protons are extruded, in part via apical Na^+/H^+ exchange, thus making HCO_3^- available for basolateral transport. In addition, apical Na^+/H^+ exchange contributes to the

generation of new HCO_3^- in the proximal tubule by extruding NH_4^+ (acid equivalent) resulting from the mitochondrial metabolism of glutamine, thereby “freeing” HCO_3^- (base equivalent) generated through the same metabolism [39–41]. Reabsorbed as well as intracellularly generated bicarbonate is extruded to the basolateral side mainly via a $\text{Na}^+-\text{HCO}_3^-$ cotransport mechanism [35, 42].

Of note, transport of solutes and water by the proximal tubule is not homogenous. The early proximal tubule reabsorbs preferentially HCO_3^- and organic solutes, and the resulting concentration of luminal Cl^- facilitates downstream paracellular Cl^- diffusion [43–46] (Fig. 1, middle). Na^+ and water reabsorption by the proximal tubule also varies axially: while fractional reabsorption of volume stays relatively constant, absolute volume reabsorption decreases along the length of the proximal tubule (linear tubular fluid to plasma inulin ratio, middle panel of Fig. 1) [43–45].

Apical Na^+/H^+ exchange is also important for proximal tubule ammonium secretion, by providing the H^+ required for luminal trapping of the freely diffused ammonia (NH_3), as well as by directly mediating $\text{Na}^+/\text{NH}_4^+$ exchange [40, 41, 47, 48]. In addition, H^+ extruded by NHE3 titrate filtered trivalent citrate to its bivalent form, thus facilitating the reabsorption of citrate via a sodium-coupled cotransport mechanism [49–51] (Fig. 1, right).

In accordance with these physiological roles, NHE3-null mice are hypovolemic and hypotensive and have mild metabolic acidosis, decreased renal reabsorption of Na^+ , fluid, and HCO_3^- , and markedly increased mortality when fed a low-salt diet [52]. These abnormalities are improved but not reversed when the expression of NHE3 is rescued by transgenic expression at the other major site of NHE3 action, the small intestine [53, 54].

Finally, apical Na^+/H^+ exchange plays a role in the proximal tubule reabsorption of filtered organic solutes such as amino acids, oligopeptides, and proteins (Fig. 2). A number of organic solute transporters in vertebrate epithelia with their evolutionary roots in prokaryotes or lower eukaryotes are H^+ -coupled rather than Na^+ -coupled [55]. In the proximal tubule, NHE3 generates the proton gradient necessary for H^+ -coupled reabsorption of filtered amino acids and oligopeptides [56, 57]. The reabsorption of filtered proteins is less well understood. Even the quantity of protein filtered by the normal glomerulus is still a matter of debate, but likely to be significant, with proximal reabsorption being a principal mechanism to render the final urine low in protein [58]. NHE3 may contribute to protein reabsorption by directly interacting with the endocytic receptor megalin at the plasma membrane [59] and by utilizing the outward Na^+ gradient of endocytic vesicles and early endosomes to drive inward movement of H^+ and endosomal acidification by approximately one pH

unit [60–63]. The role of megalin–NHE3 interaction at the plasma membrane is not clear, but from a teleological perspective it can be inferred that NHE3 is brought in the proximity of megalin before endocytosis to provide the machinery for acidification of endocytic vesicles and early endosomes. Experiments showing that megalin mediates the uptake of albumin and that albumin stimulates NHE3 abundance and turnover at the plasma membrane of cultured renal cells are compatible with this model [64, 65]. However, megalin deficiency in both rodents and humans causes low-molecular-weight proteinuria, not albuminuria [66, 67]. The current data are also insufficient to attribute the modest proteinuria of NHE3-null mice [52, 63] strictly to defective proximal tubule endosomal acidification as a de facto conclusion because of the severe extracellular fluid volume depletion and hence the high likelihood of hemodynamically related proteinuria in these animals. In addition, the role of NHE3 in proximal tubule endosomal acidification may be masked by a high degree of functional redundancy, involving the vacuolar-type H^+ -ATPase and possibly other NHE isoforms with intracellular localization (NHE6–NHE9). Additional research efforts and novel methods are required in the future to solve the quantitative debate over glomerular protein filtration [58] and to ascertain the roles of endocytic receptors, apical NHE3, and intracellular NHE isoforms in tubular protein reabsorption.

Acute vs. chronic regulation of NHE3

NHE3 is responsible for most of proximal tubule NaCl , water, and bicarbonate reabsorption, and a consequence of this high flux transport system is that relatively small percent changes in NHE3 activity can have significant quantitative and functional consequences. It is therefore not surprising that NHE3 is among the most extensively regulated membrane transport proteins, being directly or indirectly influenced by a variety of agonists and physiological conditions [68–70]. A list of factors that regulate NHE3 is presented in Table 2; please note that this list is continually evolving and is by no means exhaustive.

Before delving into further molecular intricacies, it is important to discuss the distinction between acute and chronic NHE3 regulation. Acute (minutes to hours) regulation of NHE3 is better studied, and it could be argued that it is more important for survival, by ensuring the maintenance of volume and acid–base homeostasis in the face of rapid physiological challenges. Acute regulation is via rapid, transient, and often reversible mechanisms (such as changes in phosphorylation, trafficking, or membrane locale) acting on the existing cellular pool of NHE3. Conceptually, acute regulation relies on signaling pathways which may be both saturable and refractory poststimulation, is limited in its

Table 2 Acute and chronic regulation of NHE3

| Agonist | Acute regulation | Chronic regulation | Comments | References |
|---------------------------------------|------------------|--------------------|---|--------------------------------|
| α -Adrenergic | ↑ | Unknown | Acute α -adrenergic stimulation of NHE3 occurs via the α_2 adrenergic receptor and mitogen-activated protein kinase (MAPK) activation | [67, 68] |
| Acid | ↑ | ↓ | Acid activation of NHE3 involves the autocrine–paracrine action of endothelin-1 and is enhanced by glucocorticoid hormones | [19, 69, 70, 90, 156, 174–177] |
| Adenosine | ↑ | ↓ | Acute adenosine has biphasic dosage-dependent effects on NHE3; chronically, the inhibition of NHE3 by adenosine may constitute a protective mechanism of the proximal tubule cell in ischemia | [71, 143, 178, 179] |
| Albumin | ↓ | ↓ | Albumin stimulation of NHE3 may play a role in proximal tubule protein reabsorption, but the mechanisms are not clear | [61] |
| Aldosterone | ↑ | ↑ | The acute effect of aldosterone may be cell-type-dependent: NHE stimulation was reported in brush border vesicles and cultured cells, and inhibition was reported in perfused thick ascending limbs, which express luminal NHE3 and NHE2 | [180–182] |
| Angiotensin II | ↓ / ↑ | ↑ | Angiotensin II has biphasic dosage-dependent acute effects on NHE3 and volume absorption | [80, 183–190] |
| ATP depletion | ↓ | Unknown | NHE3 does not directly utilize the energy of ATP hydrolysis, but the presence of ATP is required for optimal NHE3 activity | [191, 192] |
| Atrial natriuretic peptide (ANP) | ↓ | Unknown | ANP alone has no effect on NHE3, but it potentiates the inhibitory effect of dopamine | [193] |
| Cyclic adenosine monophosphate (cAMP) | ↓ | ↑ | Acute inhibition of NHE3 by cAMP is via activation of protein kinase A (PKA) and requires NHE3 phosphorylation at serines 552 and 605 (numbered according to the rat NHE3 sequence); other serines may also be involved | [126, 192, 194–196] |
| Dopamine | ↓ | ↓ | Both DR1-like and DR2-like receptors are involved; dopamine inhibits NHE3 via the adenylyl cyclase–cAMP–PKA pathway (above) and possibly via the PKC pathway; based on phosphopeptide mapping of NHE3, dopamine action may also involve the activation of a protein phosphatase | [82, 83, 197–200] |
| Endothelin-1 | ↑ | ↑ | Endothelin-1 regulation of NHE3 occurs via the endothelin-B receptor and likely mediates acid activation of NHE3 rather than controlling extracellular fluid volume | [156, 201–207] |
| Glucocorticoids | ↑ | ↑ | Glucocorticoids have both genomic and nongenomic actions on NHE3 (details in Fig. 4); glucocorticoids may constitute maturation signals for the NHE8-to-NHE3 developmental switch (details in Fig. 5) | [84, 91, 137, 208–211] |
| Hyperosmolarity | ↓ | ↑ | The acute response of NHE3 to hyperosmolarity is opposite to that of NHE1 and reflects the different physiological functions of the two isoforms (NHE1 is involved in cellular osmotic defense and NHE3 in whole body osmotic regulation) | [92, 138, 212] |
| Hypoosmolarity | ↑ | Unknown | In cultured renal cells, hypoosmolarity may stimulate NHE3 via changes in membrane curvature due to cell swelling; the response of NHE3 to hypoosmolarity is opposite to that of NHE1 | [72, 213, 214] |
| Insulin | ↑ | ↑ | The effect of insulin on NHE3 is biphasic and is enhanced by glucocorticoids (details in Fig. 4); the insulin effect may enhance ammonium secretion | [215–217] |
| Long-chain fatty acids | – | ↓ | The inhibitory effect may be due to lipotoxicity and lipoapoptosis; long-chain fatty acids also impair the regulation of NHE3 by glucocorticoids and insulin but not by acid | [218] |
| Lysophosphatidic acid | ↑ | Unknown | Regulation of NHE3 by lysophosphatidic acid is dependent on the scaffolding protein NHERF2 (E3KARP) | [141, 219] |
| Nitric oxide (NO) | ↓ | ↑ | Acute NO inhibits NHE3 in an intestinal cell model (Caco-2); chronic infusion of a NO synthase inhibitor in rats increases renal NHE3 abundance | [73, 108, 220] |
| Ouabain | Unknown | ↓ | Basolateral but not apical ouabain downregulates NHE3 activity and expression; decreased basolateral Na^+/K^+ -ATPase activity may signal to decrease apical NHE3 activity | [109] |

Table 2 (continued)

| Agonist | Acute regulation | Chronic regulation | Comments | References |
|--|------------------|--------------------|---|-------------------|
| Parathyroid hormone (PTH) | ↓ | ↓ | Acutely, PTH causes NHE3 phosphorylation and endocytosis via the PKA and PKC pathways | [81, 221–228] |
| Phosphatidylinositol 3,4,5-trisphosphate (PIP ₃) | ↑ | Unknown | PIP ₃ leads to rapid and reversible increase of NHE3 transport activity, while having no effect on NHE1 activity | [85] |
| Thyroid hormone | Unknown | ↑ | Thyroid hormones stimulate NHE3 by transcriptional activation and may constitute maturation signals for the NHE8-to-NHE3 developmental switch (details in Fig. 5) | [75, 76, 229–231] |

response capacity to the use of available transporters, and may overall be more costly for the cellular signaling economy by deflecting essential second messengers and protein kinases from their other intracellular roles. The chronic (hours to days) regulation of NHE3 acts in most cases via slower and more persistent mechanisms (such as transcriptional activation), provides the grounds for long-term adaptation, and may be more important for the pathophysiology of disease—such as hypertension. However, as much as acute and chronic regulations of NHE3 may differ, they are inseparable parts of the same physiological continuum.

Mechanisms of NHE3 regulation

The activity of NHE3 at the apical membrane of proximal tubule epithelial cells can be modulated via a number of mechanisms, including transcriptional [65, 71–74, 77–79] and posttranscriptional [80, 81] regulation, changes in total protein abundance, changes in protein phosphorylation [82–89], trafficking (endocytosis, exocytosis, recycling) [83, 85, 90–92], membrane locale (lipid rafts, microvilli–intermicrovillar clefts) [93–97], and the association of NHE3 with interacting proteins–complexes [70]. Only a brief summary is provided here. More extensive reviews are available [4, 70, 98, 99].

1. *Transcriptional regulation.* The 5'-flanking promoter region of the NHE3 gene contains multiple putative *cis*-acting sequences, including glucocorticoid and thyroid response elements, and consensus binding sites for various transcription factors [78, 100–102]. There are several examples of chronic NHE3 regulation by transcriptional activation, including the effects of glucocorticoids [72, 78, 79], thyroid hormones [74], insulin [73], acid [71, 77], and albumin [65]. Increased production of NHE3 transcript is usually, but not always, associated with increased total and plasma membrane NHE3 protein abundance [80, 81].
2. *Protein synthesis and degradation.* The regulation of total cellular NHE3 protein abundance at the posttranscriptional level is an important, yet underexplored area. The estimated half-life of total NHE3 protein in cultured renal cells is relatively long (~20 h) [65], and thus posttranscriptional regulation of protein abundance may play a role in chronic but not in acute NHE3 regulation. Cases of diverging regulation of total NHE3 antigen and mRNA have been reported in both rodent kidneys and cultured renal cells [80, 81], but it has been difficult to determine whether this is due to regulation of protein translation, protein degradation, or a combination of the two.
3. *Phosphorylation.* The C-terminal cytoplasmic domain of NHE3 contains multiple putative phosphorylation sites, mainly phosphoserines, and at least some of these sites are phosphorylated under basal conditions in both renal tissue and cultured cells. While the role of baseline phosphorylation is not clearly defined, there are multiple lines of evidence that additional phosphorylation and/or dephosphorylation events play a role in acute NHE3 regulation [82–88, 103, 104]. The current body of data suggests that changes in NHE3 phosphorylation occur *in vivo* [88, 105] and are *necessary* for the regulation of NHE3 by some agonists. For example, protein kinase A (PKA) activation in rats resulted in increased phosphorylation of NHE3 at serines 552 and 605 [105], and mutation of either of these serines in cultured cells abolished NHE3 regulation by the PKA activator 8-Br-cAMP [82]. However, there is evidence in both cultured cells and intact animals that phosphorylation per se is *not sufficient* for NHE3 regulation [86, 87, 104, 105]. The mechanisms by which phosphorylation alters NHE3 activity are not known. Even though it is theoretically possible that certain phosphorylation patterns may change the conformation and intrinsic transport properties of NHE3, it is more likely that phosphorylation exerts its effects by modulating NHE3 trafficking, association with regulatory proteins, or localization within the plasma membrane. Supporting

the role of phosphorylation in NHE3 trafficking, mutation of serines 560 and 613 of *Didelphis virginiana* NHE3 (equivalent to serines 552 and 605 of rat NHE3) suppressed dopamine-induced PKA-dependent changes in NHE3 plasma membrane abundance [83]. Phosphorylation of another residue conserved across mammalian species, serine 719 of rabbit NHE3, has also been recently shown to regulate NHE3 exocytic insertion into the plasma membrane [89].

4. *Trafficking.* In cultured renal epithelial cells, native NHE3 is targeted to the apical (luminal) plasma membrane, endocytosed through a clathrin-mediated pathway [106], and then returned in part to the plasma membrane via recycling endosomes [60]. Using epitope-tagged transfected protein, Alexander and coworkers [107] proposed that NHE3 exists in four subpopulations: (a) virtually immobile apical membrane NHE3, anchored to the actin cytoskeleton and dependent on Rho guanosine triphosphatase activity; (b) mobile apical membrane NHE3, not anchored to the cytoskeleton, and more likely to enter coated pits and be endocytosed; (c) rapidly recycling intracellular NHE3, presumably in recycling endosomes; and (d) a separate pool of intracellular NHE3 that exchanges slowly with the other pools. The dynamic balance between NHE3 subpopulations and the interplay between apical membrane insertion, retrieval, and endosomal recycling are what determine the amount of functional NHE3 at the apical membrane. Numerous studies have shown that NHE3 trafficking is regulated at every step, with different agonists or conditions altering NHE3 exocytosis [90–92, 108–110], recycling [65, 111], and endocytosis [83, 85]. Much less is known about the actual pathways and mechanisms involved in regulated NHE3 trafficking. What are the proteins or complexes that recruit NHE3 to exocytic vesicles? What role do posttranslational modifications play in NHE3 trafficking? What triggers NHE3 endocytosis, and what is the molecular decision-making mechanism that sends NHE3 to either recycling or degradation? Are the intracellular pools of NHE3 subject to specific regulation? These and many other questions regarding the trajectory of NHE3 within the proximal tubule cell await exploration.
5. *Redistribution along the brush border microvillus.* One potential caveat of the cell culture models in which NHE3 has been studied to date is the lack or paucity of a true brush border. Electron microscopy of opossum kidney cells, one of the most used renal epithelial cell lines expressing native NHE3, revealed the presence of scattered microvilli, in much lower numbers than in the native proximal tubule [99, 112]. Do these morphological differences influence NHE3 function and regula-

tion, and, if yes, to what extent? A series of studies by McDonough and colleagues [95–97, 113–116] have shown that different conditions or hormones that regulate NHE3 cause changes in NHE3 redistribution in density gradient fractions, which are interpreted to represent microvilli, intermicrovillar clefts, and endosomes based on cosedimentation of markers. For example, acute parathyroid hormone treatment in rats led to a shift of proximal tubule NHE3 from microvillar to intermicrovillar cleft marker-enriched fractions, and the presence of NHE3 antigen in the intermicrovillar clefts was confirmed by electron microscopy [95]. One interesting possibility is that transporter function may be regulated by redistribution along the microvillus, with NHE3 transport activity decreasing as it approaches the intermicrovillar cleft. Theoretically, this can be a kinetically driven reduction in transport due to less favorable ion gradients (lower sodium concentration and pH), a change in intrinsic transport properties due to interaction with location-specific regulatory factors, or a combination of the two mechanisms (Fig. 3). Ion gradients in the intermicrovillar region have not been directly demonstrated, but the ability of NHEs to generate a measurable spatial pH gradient was shown in vitro by single-cell electrophysiology [117]. Intermicrovillar ion gradients in vivo may be generated by transport processes and may be maintained by the difference in hydrodynamic flow between the axis of the tubular lumen and the intermicrovillar region. In addition to this conceptual model, it is possible that NHE3 in microvilli belongs to the cytoskeleton-anchored membrane NHE3 fraction, as proposed by Alexander and colleagues [107], and that actin binding is lost in intermicrovillar clefts, allowing for internalization. The interaction between NHE3 and megalin was also postulated by Biemesderfer and colleagues [59, 118] to occur in intermicrovillar clefts, where megalin-associated NHE3 is less active and awaiting endocytosis. More studies and novel methods need to be applied to resolve these mechanisms and their relative importance for NHE3 regulation.

6. *Changes in membrane locale.* The fluid mosaic model of Singer and Nicolson [119] postulated that lipids and proteins diffuse freely and are distributed randomly within cell membranes, with some restraints—such as the tight junction in epithelial cells. The current model adds another level of complexity: discrete membrane microdomains with a higher level of organization and different lipid and protein composition have been proposed to “float” in the surrounding fluid membrane and have been termed *lipid rafts* [120–122]. There are still many controversies in this field, including lipid raft size, life span, lateral mobility, potential membrane

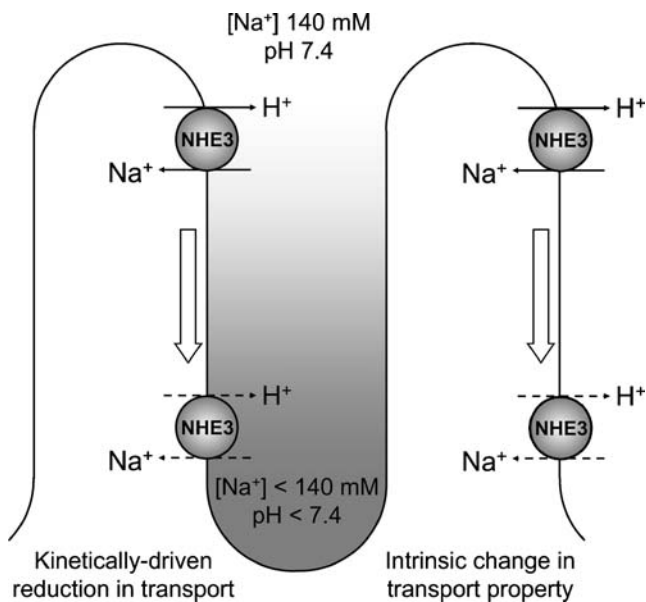


Fig. 3 Theoretical models of how apical Na^+/H^+ exchange may be regulated by relocation along microvilli in the early proximal tubule. The two models presented are not mutually exclusive and may actually coexist. A kinetically driven reduction in NHE3 activity (*left*) may be achieved by relocation toward intermicrovillar clefts, where the local ionic gradients, influenced by membrane transport processes and hydrodynamic flow, may be less favorable for Na^+/H^+ exchange. Alternatively, an intrinsic change in transport property may occur with relocation (*right*), either by detachment of NHE3 from the actin cytoskeleton or by interaction with other location-specific regulatory factors

differences between cells *in vivo* and *in culture*, and even the appropriateness of various methods used for the study of lipid rafts, but these are beyond the scope of the current discussion. Based on studies using Triton X-100 solubility, density gradients, and manipulation of membrane cholesterol content in cultured renal cells, it was reported that approximately half of apical membrane NHE3 is localized in lipid rafts and that NHE3 activity and trafficking are lipid raft dependent [93, 94]. This may be important for NHE3 regulation, as lipid rafts constitute platforms for the assembly of protein complexes and may contribute to the temporal coordination and spatial compartmentalization of membrane signaling, transport, and trafficking events [123–125].

7. *Interacting proteins.* The current knowledge about proteins interacting with NHE3 and their role in NHE3 regulation has been recently reviewed with great insight by Donowitz and Li [70]. Some of the best-studied interacting proteins, including calcineurin homologous protein, ezrin, members of the NHE regulatory factor family, and megalin, bind directly to defined regions of the C-terminal putative cytoplasmic domain of NHE3. This region confers regulatory specificity to NHE isoforms: when the cytoplasmic

domains were swapped between NHE3 and NHE1, the resulting chimeric proteins were functional Na^+/H^+ exchangers with regulatory properties similar to those of the exchangers providing the C-terminus [126, 127]. In addition to direct molecular interactions, NHE3 associates in multiprotein complexes and is tethered to the actin cytoskeleton via scaffold and adapter proteins. Specific interaction sequences with multiple protein complexes are likely to govern the strict spatiotemporal coordination necessary for cohesive NHE3 function and regulation [128].

8. *Oligomerization.* While there are substantial biochemical and structural data consistent with dimer formation of NHE1 [129–132], the data for NHE3 are rather sparse. In cultured renal cells, NHE3 associates in homodimers [133], and the pre-steady-state kinetics of proximal tubule Na^+/H^+ exchange in rabbit kidney brush border membrane vesicles are compatible with functional cooperativity within NHE3 dimers [134, 135]. Modeling of Na^+/H^+ transport kinetics using a highly sensitive electrophysiological method [117] suggests that both NHE1 and NHE3 function as coupled homodimers transporting $2\text{Na}^+/2\text{H}^+$ [136]. The role of dimerization in NHE3 regulation has not been investigated. Theoretically, NHE3 activity could be regulated via changes in dimerization, which in turn may be evoked by alterations in local membrane composition, membrane curvature, or by the competitive interaction of NHE3 monomers with other regulatory proteins. Another exciting theoretical possibility is that NHE3 dimers may constitute units of regulation (an individual regulatory signal reaching one NHE3 molecule affects the function of both molecules within the dimer), thus effectively constituting a biological amplification system for NHE3 regulation.

An integrated view of NHE3 regulation

The proximal tubule is a phenomenally complex structure, in which myriad cellular processes occur simultaneously—a fact that we sometimes tend to overlook when trying to dissect individual mechanisms. Chances are that, at any given time in the cell, NHE3 activity is affected by many factors, acting via several of the mechanisms categorized above. How is this network coordinated? Are some agonists and signaling pathways more important than others? Can we explain the molecular details of synergism, antagonism, permission, amplification, and interdependence when it comes to the simultaneous regulation of NHE3 by multiple factors—as it is the case *in vivo* in the proximal tubule?

In order to construct a model of integrated NHE3 regulation, we first need to explore and understand the

interactions between individual signaling pathways. For example, Na^+/H^+ exchange in the proximal tubule and in cultured cells expressing NHE3 is stimulated by glucocorticoids [92, 137–140] and insulin [73, 141, 142] via partly overlapping pathways (Fig. 4). In addition to activation of NHE3 transcription, the serum and glucocorticoid-inducible kinase 1 (SGK1) appears to act as a central pivot for both pathways [140, 142] and may at least in part explain the permissive effect that glucocorticoids have on insulin action [73, 142]. Another example of complex regulation is NHE3 adaptation to acidosis, which involves multiple signaling pathways and requires the autocrine–paracrine action of endothelin 1, as reviewed by Preisig [143]. A similar permissive effect of glucocorticoids has been described in NHE3 regulation by acid and has been attributed to transcriptional activation [79, 144].

Many other combinatorial patterns of NHE3 agonists likely exist in vivo, and we are still far from understanding their potential interactions. Although we have come a very long way since Pitts et al. [9, 10] first proposed a Na^+/H^+ exchange process in the kidney, we only have a distant glimpse of integrated NHE function and regulation.

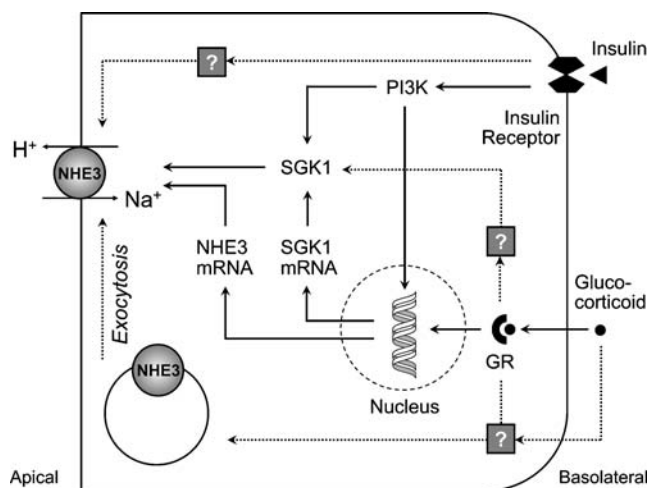


Fig. 4 Stimulation of NHE3 by insulin and glucocorticoids. *Dashed arrows* represent acute regulation (1–2 h), and *solid arrows* represent chronic regulation (>24 h). Acute regulation of NHE3 by insulin does not involve any of the known mechanisms of insulin signaling or NHE3 regulation. Chronically, insulin stimulates NHE3 via activation of phosphatidylinositol-3 kinase (*PI3K*) and serum and glucocorticoid-inducible kinase 1 (*SGK1*) and by increasing NHE3 transcription. Glucocorticoids acutely stimulate NHE3 exocytic insertion and activate SGK1 in a glucocorticoid receptor (*GR*)-dependent nongenomic fashion. Chronically, glucocorticoids regulate NHE3 via a genomic mechanism, stimulating both NHE3 and SGK1 transcription. The concerted effects of glucocorticoids and insulin on NHE3 transcription and the central role of SGK1 for both signaling pathways may at least in part explain the permissive effect that glucocorticoids have on insulin action

Luminal NHE8 and its reciprocal relationship to NHE3

Evolution has endowed mammals with multiple NHE genes. Although all NHE proteins studied to date function as Na^+/H^+ exchangers (or K^+/H^+ exchangers for some intracellular isoforms), they serve diverse functions in cells and organs. It is intuitive that each NHE isoform must possess some unique properties that justify the maintenance of more than ten different genes through mammalian evolution. Conversely, each isoform possesses sufficient versatility that it can serve more than one function. By the classification of Brett and coworkers [1], NHE8 is an intracellular isoform in most tissues but is also expressed in the proximal tubule brush border [20–22], suggesting that it may serve multiple functions. NHE3 is postulated to serve as a functional exchanger both on the plasma membrane and intracellular vesicles [60]. This type of dual function is becoming increasingly common in the NHE field. NHA2, which is much more similar to prokaryotic than eukaryotic NHEs [1], was believed to be purely an intracellular protein [145] until two recent studies demonstrated dual intracellular and plasma membrane distribution in the distal convoluted tubule, erythrocytes, and an islet cell line [7, 8]. It is highly possible that proximal tubular NHE8 serves dual functions.

NHE8 transcript is ubiquitously expressed [20, 21]. In the kidney, NHE8 protein is detected in brush border membrane vesicles by immunoblot and in the proximal apical membrane by immunohistochemistry [20–22] with some predilection for higher expression in the deep cortex [21]. Total cellular NHE8 expression is present but not increased in adult NHE3 null mice [21]. Whether apical membrane NHE8 is increased in the absence of NHE3 has not been determined, but, even if that were the case, NHE8 is insufficient to compensate for the absence of NHE3 in the proximal tubule of adult NHE3-null mice [146].

The demands on the mammalian kidney differ drastically in embryonic to neonatal and subsequently adult life. Current data support a developmental switch of NHE8 and NHE3 in the proximal tubule apical membrane. There is ample precedence for neonatal isoforms of transporters that can more aptly handle the demands of transport during that stage in life [147]. Due consideration will first be given to neonatal proximal acidification. Neonates have lower plasma HCO_3^- concentration which is predominantly due to a lower threshold for HCO_3^- absorption in the proximal tubule [148, 149]. The proximal tubular HCO_3^- threshold in turn is determined by the lower rate of neonatal proximal tubule HCO_3^- transport, which is one third that of the equivalent segment in the adult, and not by increased plasma-to-urine back leak [150, 151]. The maturational increase in proximal tubular acidification can be accounted for by the increase in apical membrane Na^+/H^+ exchange

activity [152–155]. Na^+/H^+ exchange accounts for two thirds of the luminal H^+ secretion in the adult [152, 156] and virtually all the luminal acidification in the neonate [152]. In the adult, NHE3 is the predominant Na^+/H^+ exchanger mediating proximal tubule acidification [19, 52, 157]. Indeed, there is a parallel increase in Na^+/H^+ exchanger activity [152, 154, 155] and NHE3 protein and transcript with maturation [153]. However, changes in NHE3 expression alone could not explain all the findings.

There is considerable Na^+/H^+ exchanger activity in neonatal rats despite very low levels of NHE3 protein on brush border membranes [158, 159]. In 30-day-old rats subjected to adrenalectomy to arrest maturation, there is substantive amount of Na^+/H^+ exchanger activity despite the distinct paucity of NHE3 protein on the apical membrane [159]. In the NHE3 and NHE2 double-knockout mice, there was clearly residual luminal Na^+/H^+ exchanger activity in the proximal tubule [160]. The collective data support another NHE on the apical membrane, especially in the neonate, that mediates renal acidification. Based on its renal localization, it is likely that NHE8 is the neonatal proximal tubule NHE isoform.

The induction of the NHE8-to-NHE3 developmental switch (Fig. 5) can be intrinsic to the kidney, secondary to circulating factors, or both. To date, evidence exists only for endocrine control. There is a threefold increase in thyroid hormone level and a 25-fold increase in corticoste-

rone level in rat plasma during postnatal maturation [161–164] and these hormonal surges mirror the maturational increase of NHE3 [159]. Perinatal increases in glucocorticoids and thyroid hormone have been proposed by Baum and coworkers [151, 153, 164, 165] to be responsible for the postnatal increase in proximal tubule NHE3 and acidification, and causality is supported by several observations. Administration of glucocorticoids and thyroid hormone prior to the developmental increase in serum levels results in acceleration of the maturation of Na^+/H^+ exchanger activity and NHE3 mRNA and NHE3 protein abundance [151, 153, 159, 164]. In cultured cells, both glucocorticoids and thyroid hormone directly increase NHE3 transcription and protein abundance [72, 74]. In addition, posttranslational protein trafficking is also involved in the increase in surface NHE3 protein by glucocorticoids [92, 166]. Conversely, preventing the maturational increase in either thyroid hormone or glucocorticoids significantly suppresses the maturational increase in Na^+/H^+ exchanger activity and brush border membrane NHE3 protein [159, 164]. Experimentally induced hypothyroidism delays the maturational increase in NHE3 and prolongs the expression of neonatal NHE8 [167]. Interaction and complementation between thyroid hormone and glucocorticoid levels have been well described [168–171]. Interestingly, the reduction in NHE3 protein is not accompanied by changes in NHE3 mRNA abundance, once again highlighting the multilevel regulation of NHE3.

NHE3 protein in total cortex and apical membrane increases concordantly with maturation [153]. In contrast, while apical membrane NHE8 decreases dramatically upon maturation, its level in total cortical membranes is higher in the adult compared to the neonatal proximal tubule [22]. Native NHE8 in cultured cells also has considerable intracellular expression [172]. One possible paradigm is that NHE8 serves primarily as an organellar exchanger in adults, but, in neonates prior to the maturation of NHE3 on the brush border, NHE8 is temporarily “leased” to the apical membrane to perform transepithelial transport.

As mentioned above, Brett and coworkers [1] predicted NHE8 to be an intracellular protein. The function of intracellular NHE8 is unclear at present. Transport characteristics for NHE8 may be more suited for the neonatal proximal tubule than NHE3. Na^+ kinetics for example exhibits a higher degree of cooperativity than NHE3 [172] which is what one may expect for an intracellular NHE utilizing vesicular Na^+ to drive intravesicular acidification. Nakamura and coworkers [173] found evidence for K^+/H^+ exchange for NHE8 when reconstituted in proteoliposomes but plasma membrane NHE8 in mammalian cells does not seem to have such properties [172]. Capability of K^+/H^+ exchange may be intrinsic for the protein but not functional in the plasma membrane environment. A most important

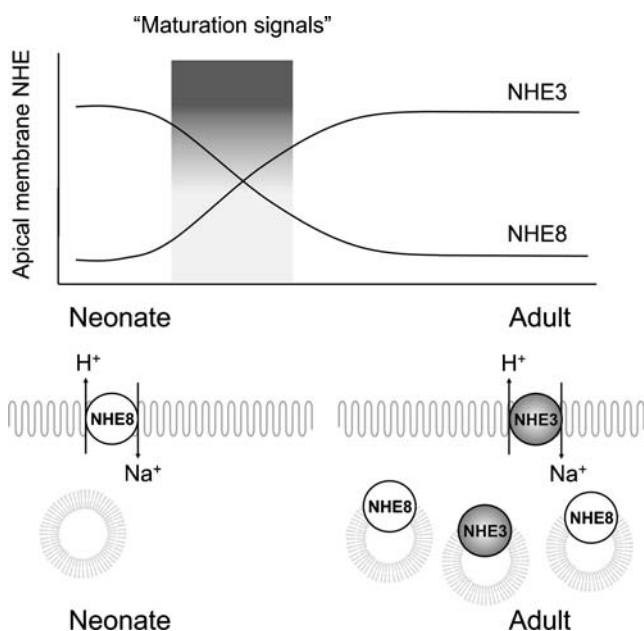


Fig. 5 Developmental switch of NHE8 and NHE3 at the proximal tubule apical membrane. Maturation signals can be from the systemic circulation, intrinsic to the kidney, or both. While there is parallel increase in total cellular and apical membrane NHE3 upon maturation, the decrease in apical NHE8 in the adult is accompanied by increase in intracellular NHE8

and challenging question is what role does intracellular NHE8 play.

Summary

This account covers the role of luminal Na^+/H^+ exchangers in proximal tubule function and updates selected aspects of the current database on the mechanisms of regulation of NHE3 that are not detailed in other more extensive reviews. Mammals are endowed with multiple NHE genes and have kept this genetic inventory over many millennia without extinguishing any of these sequences. This suggests an “insurance policy” of high redundancy, unique irreplaceable properties of each isoform, or both. Conversely, individual isoforms such as NHE3 participate in extremely diverse functions, such as those described here at the lumen of the proximal tubule. Comparable to its scope of function, the levels of regulation of NHE3 are astoundingly broad—and this is just NHE3 in the proximal tubule, our *hors d'oeuvre* so to speak, in this field. One has not even begun to consider all the other NHE isoforms in all other nephron segments. The database is of course still very much empty and awaits discovery. NHEs provide a myriad of functions critical for renal physiology in each segment—transport of solutes such as NH_4^+ within the complex architecture of the medullary, the role of intracellular NHEs for organellar function, nontransport functions as plasma membrane platforms for protein assembly—tasks immense enough to fill several generations of investigators.

The authors took the liberty to linguistically interpret this *molecularly guided tour along the nephron* conceived by the editors as one that places equal emphasis on the molecule as well as the nephron. There is no doubt that the awesome power of recombinant DNA techniques has spawned a revolution and explosion of knowledge on renal physiology with unprecedented speed and scope; an effect whose momentum will not stall but rather continue to accelerate. All this is triumphant news for the renal physiologist. However, it will be deceiving to assume and believe that the understanding of individual molecules in exquisite detail and precision automatically begets the understanding of tubular, renal, and whole-organism biology. There is a hierarchy from individual molecules to the cell, epithelia, nephron, kidney, and eventually for a physiologist, the organism. At each level of this pyramid, new complexities, patterns, and laws emerge. Such properties cannot be studied and understood when outfitted solely with knowledge derived from a different level. A translated citation of Claude Bernard embodies this spirit eloquently: “We must appreciate that when we break up an organism by taking the different components apart it is only for the sake of convenient experimentation and by no means because

we consider them as separate entities. Indeed when we wish to ascribe to a physiologic property its significance we must always refer it to the whole organism and draw any conclusions only in relation to the effect of this property on the organism as a whole.”

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