Physiological and Molecular Function of the Sodium/Hydrogen Exchanger NHA2 (SLC9B2)

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Abstract: NHA2, also known as SLC9B2, is an orphan intracellular Na\(^+\)/H\(^+\) exchanger (NHE) that has been associated with arterial hypertension and diabetes mellitus in humans. The objective of this NCCR TransCure project was to define the physiological and molecular function of NHA2, to develop a high resolution kinetic transport assay for NHA2 and to identify specific and potent compounds targeting NHA2. In this review, we summarize the results of this highly interdisciplinary and interfaculty effort, led by the groups of Proffs. Jean-Louis Reymond, Christoph von Ballmoos and Daniel Fuster.

Keywords: Blood pressure homeostasis · Bone · β-Cell · Insulin secretion · Kidney · Na\(^+\)/H\(^+\) exchanger · NHA2 · NHE · Osteoclast · SLC9B2

1. Introduction

1.1 Na\(^+\)/H\(^+\) Exchangers, a Conserved Family of Membrane Transport Proteins Involved in Regulation of Cytoplasmic and Organellar pH

Na\(^+\)/H\(^+\) exchangers (NHEs) are ion transporters present in lipid bilayers in simple prokaryotes and eukaryotes, including plants, fungi and animals which harness the electrochemical gradient of one ion to energize the uphill transport of the other.[1] In mammals, 13 evolutionarily conserved NHE isoforms are currently known.[2] Human NHEs are encoded by the SLC9 gene family (solute carrier classification of transporters: www.genenames.org), which is divided in three subgroups.[2] The SLC9A subgroup encompasses plasmalemmal isoforms NHE1-5 (SLC9A1-5) and the predominantly intracellular isoforms NHE6-9 (SLC9A6-9). The SLC9B subgroup consists of the two recently cloned isoforms NHA1 and NHA2 (SLC9B1 and SLC9B2, respectively), which possess higher similarity to prokaryotic NHEs than the other members of the SLC9 family.[1] The SLC9C subgroup consists of a single gene encoding plasmalemmal NHE (SLC9C1) and a putative NHE SLC9C2 for which no functional data exist so far.[2]

The steady-state pH of different intracellular compartments varies greatly in mammalian cells but they are tightly controlled[3] (Fig. 1). In the endocytic pathway, vesicular pH gradually decreases in an inbound direction from early endosomes to lysosomes, with the latter exhibiting a pH of 4.5–5.[4] This graded acidification is essential for recycling and/or degradation of internalized membrane proteins and fluid-phase solutes.[3] Similarly, the progressive acidification of vesicles along the secretory pathway is important for proper post-translational processing, sorting and transport of newly synthesized proteins.[5] The intraluminal pH of vesicles and organelles is the result of acidification by the ATP-driven V-ATPase, which in turn is counteracted by H\(^+\) leak pathways.[4,6] A large fraction of mammalian NHEs is devoted to pH regulation of intracellular vesicles and organelles. However, the individual contributions of these intracellular NHE paralogues to organellar function and vesicular trafficking are still...
To study the role of NHA2 in bone homeostasis and more specifically in osteoclast differentiation and bone resorption, we generated two different strains of NHA2 knock-out (KO) mice. Mutant mice of both strains were born at expected Mendelian ratios, developed normally and appeared healthy without obvious phenotype. Detailed studies with osteoclasts derived from wild-type (WT) and NHA2 KO mice revealed normal differentiation of NHA2-deficient osteoclast precursors to mature osteoclasts and normal resorptive function of mature osteoclasts lacking NHA2 in vitro. Structural parameters of bone, quantified by high-resolution microcomputed tomography (μCT), were not different between WT and NHA2 KO mice. Also when osteoclast differentiation was stimulated in vivo by ovariectomy in female mice, no differences in bone loss between WT and NHA2 KO mice were detectable. These findings were later confirmed independently by another group using a different NHA2 KO mouse strain and together indicate that NHA2 is dispensable for osteoclast differentiation and bone resorption.

When performing comparative expression studies of NHEs, we discovered significant upregulation of NHA1 (SLC9B1) in NHA2-deficient osteoclasts, suggesting possible functional compensation by this closely related SLC9B isoform. We recently generated NHA1 KO and NHA1/NHA2 double KO mice to further study interaction and function of the two SLC9B transporters in bone cell biology.
2.2 NHA2 is Critical for Insulin Secretion in β-Cells of the Endocrine Pancreas

Given the possible association of NHA2 with diabetes in humans,[8,10,11,17,18] we investigated the role of NHA2 in systemic glucose homeostasis. We discovered that NHA2 is expressed in human as well as rodent β-cells and β-cell lines.[14] Knock-down of NHA2 in the murine β-cell line Min6 reduced both glucose- and sulfonylurea-induced insulin secretion. Overexpression of WT but not functionally-dead human NHA2 rescued the insulin secretion deficit induced by knock-down of endogenous NHA2 in Min6 cells. Similar findings were obtained in vitro when we studied islets of two different NHA2 KO strains. Also heterozygous mice of both lines exhibited an insulin secretion deficit. In vivo, we found that both strains of NHA2 KO mice displayed a pathological glucose tolerance with impaired insulin secretion but normal peripheral insulin sensitivity. Both pancreatic and islet insulin and proinsulin contents were not different between WT and NHA2 KO mice, suggesting that impaired insulin synthesis or maturation are not the cause of the insulin secretion deficit. Subcellular fractionation and imaging studies revealed that NHA2 resides predominantly in endosomes, and clathrin-dependent endocytosis was significant—lysine kinase 4 (WNK4) at its core.

Transferrin-positive endosomes (early and recycling endosomes). and imaging studies revealed that NHA2 resides predominantly in other cellular PKC and PKA substrates was not affected. Also, loss of NHA2 did not affect levels of active, phosphorylated PKA or PKC isoforms responsible for KLHL3 phosphorylation nor the abundance of the calcium-sensitive phosphatase calcineurin, very recently shown to be involved in KLHL3 dephosphorylation.[34]

In a follow-up publication, we demonstrated that loss of NHA2 greatly exacerbated aging- and obesity-induced glucose intolerance in mice.[19] In support of these findings, a single-nucleotide polymorphism in the NHA2 gene (rs4699049) was recently discovered in a genome-wide association study as a new locus associated with type 2 diabetes (and renal function) in humans.[20]

Together these results reveal that NHA2 is critical for clathrin-dependent endocytosis and insulin secretion in β-cells. Exocytosis and endocytosis are tightly coupled in β-cells, and inhibition of endocytosis by various approaches reduces insulin secretion.[17,21–23] We thus speculate that the insulin secretion deficit observed upon loss of NHA2 is due to disrupted endo-exocytosis coupling. Clearly, however, the exact role of NHA2 in the endosome of β-cells remains unknown and is currently under investigation.

2.3 NHA2 Regulates Blood Pressure Homeostasis and Electrolyte Handling in the Kidney

We previously discovered that NHA2 exhibits significant expression in the kidney, and reported the localization of NHA2 to the distal nephron.[29] In follow-up experiments, we further defined the expression profile along the nephron in the mouse kidney. Our studies revealed that expression levels of NHA2 were by far the highest in distal convoluted tubules (DCT).[24] DCT cells express the apical thiazide-sensitive Na+/Cl− cotransporter NCC (also known as SLC12A3), a key transporter responsible for Na+ and Cl− reabsorption and hence regulation of extracellular volume and blood pressure in mammals. NCC activity is regulated by an intracellular kinase network with the serine-threonine with-nosyline kinase 4 (WNK4) at its core.[25] Once activated, WNK4 phosphorylates the Ste20/SPS1-related protein alanine-rich kinase (SPAK), which in turn phosphorylates and activates NCC. WNK4 abundance and activity are tightly controlled by posttranslational modifications. An E3-RING ubiquitin ligase complex that includes the two regulatory proteins Cullin 3 (CUL3) and Kelch-like 3 (KLHL3), ubiquitylates WNK4 and thereby induces its proteasomal degradation. Protein kinase C (PKC) and protein kinase A (PKA), activated by angiotensin II and vasopressin, respectively, directly phosphorylate and activate WNK4.[26] In addition, PKC and PKA were also shown to phosphorylate KLHL3, which leads to disruption of the KLHL3–WNK4 interaction and hence prevents WNK4 ubiquitylation.[27,28]

The DCT is paramount for blood pressure homeostasis in mammals. Biallelic loss-of-function mutations or deletions of NCC result in Gitelman’s syndrome, characterized by hypotension, hypokalemia, metabolic alkalosis and hypocalciuria.[29,30] In contrast, dominant mutations in WNK1, WNK4, CUL3 or KLHL3 cause the mirror phenotype, pseudohypoaldosteronism type II (PHA II, also known as Gordon’s syndrome), characterized by hypertension, hypokalemia, metabolic acidosis and hypercalciuria.[31–33]

Phenotypic studies revealed that NHA2 KO mice exhibit features resembling Gitelman’s syndrome, including low blood pressure, normocalcemic hypocalciuria and a blunted response to thiazide diuretics (=NCC inhibitors). Our extensive in vitro and in vivo studies revealed that loss of NHA2 leads to a cell-autonomous, specific downregulation of the WNK4 - SPAK - NCC axis in DCT cells. Phenotype analysis of NHA2/NCC double KO mice further supported the notion that NHA2 affects blood pressure homeostasis by an intrarenal and NCC-dependent mechanism. Loss of NHA2 results in reduced WNK4 levels, caused by increased WNK4 ubiquitylation due to reduced PKC- and PKA-mediated phosphorylation of KLHL3, an E3-RING ubiquitin ligase complex protein involved in WNK4 ubiquitylation. Phosphorylation of other cellular PKC and PKA substrates was not affected. Also, loss of NHA2 did not affect levels of active, phosphorylated PKA or PKC isoforms responsible for KLHL3 phosphorylation nor the abundance of the calcium-sensitive phosphatase calcineurin, very recently shown to be involved in KLHL3 dephosphorylation.[34]

In support of these results, a recent study demonstrated that a high Na+ diet results in augmented NHA2 expression in the mouse kidney, which is consistent with a role of NHA2 in renal Na+ handling and blood pressure regulation.[35] Similarly, the Drosophila orthologues of the two mammalian SLC9B transporters (Nha1 and Nha2) are expressed in renal tubules and are induced by a high Na+ diet.[36]

Thus, NHA2 plays an important role in renal electrolyte handling and blood pressure regulation. However, the molecular mechanisms of how NHA2 ultimately regulates NCC activity in DCT cells remain unclear and are currently being actively investigated in our laboratory.

3. Identification of Novel Highly Potent and Selective NHA2 Inhibitors

The pharmacology of intracellular NHEs is unexplored, and no specific compounds targeting SLC9B transporters are known. We developed a yeast-based functional complementation assay adapted to 96-well plates to measure NHA2 function and enable screening of compound libraries.[9] Heterologously expressed human NHA2 rescues the salt-sensitive growth phenotype of the Saccharomyces cerevisiae strain AXT3 (enal– 4 nha1 nha1) that lacks major Na+ and Li+ handling ion transporters. The host strain is unable to grow in the presence of high extracellular Na+ or Li+ but transformation with WT NHA2 (but not mutant functionally-dead NHA2 or the empty vector) rescues the salt-sensitive phenotype (Fig. 3A). To search for potent NHA2 inhibitors, we performed an in silico virtual screen based on a 3D-pharmacophore and shape-similarity scoring function developed in the context of a different TransCure project.[37] Starting with the bacterial NhaA inhibitor 2-aminopyrimidine and the amiloride derivative 5-(N,N-hexamethylene) amiloride as reference compounds, we virtually screened a commercial library of 800,000 drug-like molecules and eventually selected and purchased 63 compounds which were tested in the yeast assay. After detailed structure–activity relationship (SAR) studies and repeat screening, we identified a series of very potent NHA2 inhibitors with IC50 values in the low nM range. These inhibitors were non-toxic to yeast and mammalian cells up to 100 µM concentration, and did not inhibit plasma membrane paralogues NHE3 and NHE1 or endosomal paralogues NHE6 and NHE9. In a next step, we tested the most potent inhibitor C3hn064 with primary islets to inhibit insulin secretion. As shown in Fig. 3B, C3hn064 caused a dose-dependent inhibition of insulin se-
cretion in islets of WT mice. Maximal inhibition occurred at low µM concentrations to secretion levels comparable to islets isolated from NHA2 KO mice. Throughout the concentration range tested, Cjnh064 did not inhibit insulin secretion from NHA2 KO islets (Fig. 3B). IC_{50} of Cjnh064 for insulin secretion excretion in islets (40–50 nM) was in accordance with results obtained in the yeast assay. Detailed in vitro profiling studies and pharmacokinetic experiments in rodents are currently ongoing.

4.1 Expression and Purification of NHA2 from HEK293 Cells

Using the Flp-In™ T-REx™ system, stably transfected HEK293 cell lines were generated that allowed doxycycline-inducible expression of 3xFLAG-NHA2 fusion proteins. The 3xFLAG affinity tag was placed at the N-terminus or C-terminus of both wild-type and functionally inactive NHA2 and can be removed using HRV3C cleavage. Inducible expression of NHA2 WT and the NHA2 DDNN mutant fusion proteins in HEK293 cells was verified by immunofluorescence microscopy and immunoprecipitation experiments (Fig. 4A, B). The N-terminal tagged protein showed fewer degradation products and was used for large-scale expression using the Nunc™ Cell Factory™ system. From the obtained cell material, NHA2 was extracted from membranes using either detergent (DDM and LMNG) or amphipathic styrene-maleic acid (SMA) copolymer and purified via affinity chromatography (Fig. 4C, D). Addition of SMA to biological membranes leads to the spontaneous formation of Discoi- dal structures known as SMA-Lipid Particles (SMALPs) that were shown to extract proteins with their annular layer of lipids, improving protein stability. Both approaches yielded NHA2 that was eluted from the affinity column and verified by mass spectrometry. A single band was obtained using SMA, while two lower running bands were observed in the detergent-based purification that were identified as partially degraded NHA2 and Actin G. Both purification strategies resulted in a rather low protein yield compared to heterologous expression and purification of human NHA2 in yeast, but sufficient to initiate liposome transport measurements.
4.2 Co-reconstitution of ATP Synthase and NHA2 into Liposomes to Follow Transport Kinetics

We utilized a now well-established assay in field, that was developed to measure Na⁺/H⁺ antiport with NapA from Thermus thermophilus.[38] Here, purified F₁,Fₒ ATP synthase from E. coli is co-reconstituted with the antiporter into liposomes, and the inner lumen of the vesicles is acidified by ATP-driven proton influx that is followed by ACMA fluorescence quenching. Addition of Na⁺ or Li⁺ activates antiport, driving Na⁺ ions into the liposomes and H⁺ efflux that is observed as change in fluorescence (Fig. 5A). Alternatively, antiporter activity can also be followed in the absence of ATP synthase.[38] Here, the protein is reconstituted into liposomes loaded with the pH-sensitive fluorophore pyranine. Transport activity is initiated by establishment of a proton or sodium motive force as described for NapA earlier (Fig. 5E).[38] Lipids are expected to play a crucial role for protein stability, transport activity, and enzyme regulation. For human NHA2, we have thus utilized a lipid composition similar to what is found in the late endosome, supplemented with multiple phosphatidylinositol phosphates (PIP). PIP, and PIP₃, that have been shown to stabilize the homodimeric state of Equus caballus NHE9.[41] Recent studies with a truncated bison NHA2 variant (residues 70–554) demonstrated that PIP₃ has a thermostabilizing effect on the enzyme. Native mass spectroscopy and thermostability analysis showed that PI lipids bind specifically to the bison NHA2 variant and their presence further stabilized the native homodimer.[42]

In short, purified protein (WT and DDNN mutant) was incubated with preformed liposomes (100 nm) in the presence of octyl glucoside and excess detergent was removed using Bio-Beads. The liposomes were collected via ultracentrifugation, resuspended in fresh buffer and successful insertion into liposomes was verified using SDS PAGE followed by Western Blot (Fig. 5B). For SMA purified protein, a recently described protocol using sonicating for the reconstitution of yeast cytochrome c oxidase was employed.[43]

4.3 Kinetic Transport Assays of NHA2 Proteoliposomes

A representative selection of single transport kinetics is depicted in Fig. 5C–F. In co-reconstitution experiments with ATP synthase, a decrease in fluorescence is observed upon addition of ATP, indicating acidification of the liposomes. After establishment of a new equilibrium between pumping and liposome leakage, 45 mM Li₂SO₄ was added, and a rapid increase to a new equilibrium is observed in the control with NapA (Fig. 5C, orange trace). A negligible signal increase is observed in vesicles containing full-length human NHA2 (Fig. 5C, blue trace), and the same signal is observed for the inactive DDNN mutant (Fig. 5C, red trace), suggesting that the reconstituted enzyme is inactive. This is nevertheless noteworthy, as an earlier, similar experiment with truncated NHA2 purified from yeast showed a stronger signal for both WT (Fig. 5D, blue trace) and inactive DDNN variant (Fig. 5D, red trace), suggesting that unspecific transport took place with the truncated variant. It should be noted that the amount of reconstituted NHA2 purified from yeast was ~10x higher than from HEK293 cells. We also followed Na⁺/H⁺ antiport activity in liposomes containing NHA2 and 60 mM Na₂SO₄ that were diluted into Na⁺-free buffer, initiating Na⁺-export and H⁺ import. The resulting acidification was followed using liposome entrapped pyranine (Fig. 5E). In contrast to the co-reconstitution experiments, acidification was observed with both WT (Fig. 5F, blue trace) and the inactive DDNN mutant (Fig. 5F, red trace), suggesting again unspecific transport. We have further varied lipid composition, protein purification method, and driving force, however, we were unable to substantially robust NHA2 activity for the WT and absence of activity for the DDNN mutant. It is difficult to find conclusive reasons for the absence of activity, as our assays are sensitive enough to also follow even low transport rates. On the other hand, it is not uncommon to find unspecific transport activities in the presence of externally applied ion or electrical gradients across liposomal membranes, making controls with an inactive enzyme critically relevant.

5. Conclusions

NHA2 exhibits high expression in β-cells of the endocrine pancreas and is critical for insulin secretion and systemic glucose homeostasis. In the kidney, NHA2 is confined to specialized tubular cells of the distal convolution where it regulates Na⁺ reabsorption and thereby blood pressure. In contrast, NHA2 is dispensable for osteoclast differentiation, bone resorption and bone turnover. Studies to further define the molecular function of NHA2 at a subcellular level are ongoing. By combining an in silico virtual screening approach coupled with an in-house developed yeast-based functional complementation assay to quantify NHA2 transport function, we discovered several specific and highly potent NHA2 inhibitors that are currently further characterized in preclinical studies. Overexpressed human NHA2 was successfully purified from large scale expression cultures and reconstituted in proteoliposomes. We are currently working to improve reconstitution conditions and hence functional read-outs to study NHA2 kinetics and characterize our NHA2 inhibitors.