Lessons from Mouse Mutants of Epithelial Sodium Channel and Its Regulatory Proteins

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The use of gene-modified mouse models allows the experimental in vivo analysis of specific gene defects at the level of target cells. With respect to the epithelial sodium channel and some of its regulatory proteins, gene-modified models that control gene defects in a time- and tissue-dependent conditional or constitutive manner have been generated. The combination of molecular and physiologic approaches in these mouse models increases the understanding of the complex regulation and the cell signaling cascades involved in Na⁺ transport in target cells and may ultimately provide new insights into the pathophysiology of renal Na⁺ retention and BP regulation. This review summarizes and discusses the gene-targeting approaches that have been applied to the epithelial sodium channel and its regulatory proteins.


The amiloride-sensitive epithelial sodium channel (ENaC) is an apical cell membrane constituent of many salt-absorbing epithelia. In the kidney, the functional relevance of ENaC for the aldosterone-dependent Na⁺ reabsorption in the “aldosterone-sensitive” distal nephron (ASDN) and thus for the regulation of extracellular volume and BP is well established (for review, see 1). Important evidence derived from patients in which mutations in the SCNN1 genes encoding ENaC subunits result in renal salt retention (gain-of-ENaC function mutation) or renal salt loss (loss-of-ENaC function mutation) and thus are causative for Liddle’s syndrome, a severe form of human hypertension, or pseudohypoaldosteronism (PHA-1), a salt-wasting hypotensive syndrome, respectively. In this review, we discuss the significant insights into the role of ENaC and its regulatory proteins in renal function and electrolyte and extracellular volume homeostasis provided by genetic mouse models.

Loss-of-Function ENaC Mutants

Gene inactivation studies for all three subunits of ENaC (α, β, and γ; encoded by Scnn1a, Scnn1b, and Scnn1g, respectively) revealed a crucial role for each subunit in survival of the animal. Constitutive inactivation of α-ENaC demonstrated the important role of the channel in lung liquid clearance after birth, i.e., mice that lack α-ENaC die within 40 h after birth because impaired lung liquid clearance prevents normal ventilation and respiration (2) (Table 1). These mice show a complete abolishment of ENaC activity in airway epithelia, suggesting that βγ subunits alone do not confer sufficient activity to compensate for loss of α-ENaC. Furthermore, α-ENaC knockout mice present metabolic acidosis with lower blood pH and low bicarbonate concentrations, suggesting a metabolic component added to the probable respiratory acidosis (2). Further insights were provided by ENaC transgenic rescue mice (Scnn1d<sup>tm<sup>1</sup>tm<sup>3</sup></sup>TgraENaC) that express low constitutive α-ENaC under the control of a heterologous cytomegalovirus (CMV) promoter in an α-ENaC genetic knockout background (3) (Table 1). Fifty percent of these mice exhibit clinical features of a severe PHA-1 and die within 2 wk after birth. The survivors exhibit a compensated PHA-1 with normal acid/base and electrolyte values but sixfold elevated plasma aldosterone levels (3–5). Although apparently competent for airway fluid clearance, these same mice present constitutively impaired transepithelial Na⁺ transport in the lung, which is also found in patients with PHA-1 (6), and the mice provided evidence that this defective respiratory transepithelial Na⁺ transport may facilitate pulmonary edema (7). Moreover, when downregulation of ENaC activity (e.g., observed under hypoxia) is imposed on a low constitutive ENaC expression, the resulting reduced Na⁺ transport rate may become insufficient for airway fluid clearance (8,9). In comparison, heterozygous mutant mice for α-ENaC have no lung phenotype and show an intact capacity to maintain BP and Na⁺ balance, although plasma renin activity did not change when studied on normal and low NaCl diets. They exhibit an increased vascular responsiveness to exogenous angiotensin II,
and their BP is lowered markedly during angiotensin II receptor blockade, indicating a compensatory upregulation of angiotensin type 1 receptors (10).

β-ENaC and γ-ENaC null mutants exhibit early renal dysfunction that leads to death within 48 h (4,5) (Table 1). Lethargy and failure to thrive are associated with urinary Na⁺ wasting, K⁺ retention, and increased plasma aldosterone concentrations, thus reflecting the renal phenotype found in patients with PHA-1. Conversely, low residual ENaC activity in these mice is sufficient to circumvent the neonatal lung phenotype, consistent with the assumption that αβ and αγ subunit combinations can establish some ENaC activity in airway epithelia.

In the course of generating a mouse model for Liddle’s syndrome by the insertion of a stop codon (corresponding to residue R566 in human β-ENaC) and the selection marker neomycin, we obtained mice with a disruption of the ENaC gene locus (Scnn1bneo/neo) (11). These mice show a reduced ENaC activity in vitro and failure to thrive are associated with urinary Na⁺ wasting, hyperkalemia, and acid-base homeostasis (Figure 1). Variations in the time of onset and severity of symptoms can be noticed between these models (β-ENaC−/−, γ-ENaC−/−, Scnn1bneo/neo).

### Table 1. Phenotypes of mouse mutants of ENaC and its regulatory proteins

<table>
<thead>
<tr>
<th>ENaC Alleles and Genotype</th>
<th>ENaC Activity (%)</th>
<th>Phenotype</th>
<th>Affected Organs</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Null alleles</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scnn1a&lt;sup&gt;tm1/bml&lt;/sup&gt;</td>
<td>0</td>
<td>perinatal lethality</td>
<td>lung, kidney</td>
<td>(2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>skin</td>
<td>(45)</td>
</tr>
<tr>
<td>βENaC−/−</td>
<td>ND</td>
<td>perinatal lethality</td>
<td>kidney</td>
<td>(5)</td>
</tr>
<tr>
<td>γENaC−/−</td>
<td>&lt;15</td>
<td>perinatal lethality</td>
<td>kidney</td>
<td>(4)</td>
</tr>
<tr>
<td>Mutant alleles</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scnn1b&lt;sup&gt;neo/neo&lt;/sup&gt;</td>
<td>approximately 20</td>
<td>PHA-1</td>
<td>kidney</td>
<td>(11)</td>
</tr>
<tr>
<td>Scnn1b&lt;sup&gt;Lid/Lid&lt;/sup&gt;</td>
<td>&gt;100</td>
<td>Liddle’s syndrome</td>
<td>kidney</td>
<td>(23,24,25)</td>
</tr>
<tr>
<td>Scnn1a&lt;sup&gt;lox/lox&lt;/sup&gt;</td>
<td>100</td>
<td>normal</td>
<td>—</td>
<td>(18)</td>
</tr>
<tr>
<td>Transgenic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scnn1a&lt;sup&gt;lox/lox&lt;/sup&gt;TgraENaC</td>
<td>approximately 15</td>
<td>PHA-1</td>
<td>kidney</td>
<td>(3)</td>
</tr>
<tr>
<td>Scnn1a&lt;sup&gt;lox/lox&lt;/sup&gt;Hoxb7Cre</td>
<td>±0 to 100</td>
<td>pulmonary edema</td>
<td>lung</td>
<td>(7,8)</td>
</tr>
<tr>
<td>Regulatory proteins of ENaC</td>
<td></td>
<td></td>
<td>normal</td>
<td></td>
</tr>
<tr>
<td>Null alleles</td>
<td></td>
<td></td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Prss10&lt;sup&gt;lox/lox&lt;/sup&gt;</td>
<td>100</td>
<td>normal</td>
<td>—</td>
<td>(44)</td>
</tr>
<tr>
<td>Scnn1a&lt;sup&gt;lox/lox&lt;/sup&gt;</td>
<td>approximately 55&lt;sup&gt;b&lt;/sup&gt;</td>
<td>PHA-1</td>
<td>kidney, brain</td>
<td>(34,35,37)</td>
</tr>
<tr>
<td>MR−/−</td>
<td>approximately 25</td>
<td>postnatal lethality</td>
<td>kidney</td>
<td>(28,29)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Estimated from in vitro or in vivo experiments.

<sup>b</sup>On low salt diet.

These variations may be due at least in part to the fact that none of the identified human PHA-1 ENaC mutations is a null mutation and that at birth the kidney development is more complete in human than in mouse.

Besides the classical gene-targeting approach, whereby part of the gene encoding the protein is removed or destroyed, conditional gene targeting is increasingly used to evaluate the role of a gene/protein of interest in a specific cell type and/or a time-dependent manner. Gene targeting of a particular segment of the nephron is dependent on the choice of promoters used to target the expression of Cre recombinase to a distinct cell type within one or more segments along the nephron in vivo. Transgenic mice with renal cell type–specific expression of Cre recombinase can be generated and used subsequently, for example, to induce inactivation of a gene in a specific nephron segment (14–17). Recently, a more refined mouse model that allows expression of specific ENaC mutations in a time- and tissue-specific manner has been developed (18). These mice contain a floxed Scnn1a allele, and the coding sequences (exon 1) can be removed efficiently by Cre recombinase in vivo. Complete absence of this exon 1 leads to a lethal phenotype as described for α-ENaC deficiency by Scnn1a<sup>tm1</sup> allele (18) (Table 1). Using the floxed Scnn1a (α-ENaC; Scnn1a<sup>lox/lox</sup>) allele, we generated mice in which α-ENaC expression was selectively abolished in cortical collecting duct (CCD) but not in the early segments of the ASDN, namely late distal convoluted tubule (DCT) and connecting tubule (CNT) (16). Most important, this inactivation did not impair Na⁺ and K⁺ balance or induce a salt-wasting phenotype. The animals survive well and show...
normal water, Na\(^+\), and K\(^+\) balance, even when challenged by water deprivation, K\(^+\) loading, and salt restriction with plasma aldosterone concentrations not different from wild-type mice. We suggest that expression of ENaC in the CCD is not a prerequisite for normal Na\(^+\) and K\(^+\) balance in mice, pointing to the importance of ENaC activity in the early ASDN (16). In fact, there is an increasing body of evidence that late DCT and CNT, rather than CCD, are the main physiologic regulators of urinary Na\(^+\) and K\(^+\) excretion. ENaC-mediated currents decrease in magnitude between CNT, the initial part of CCD, and CCD in aldosterone-treated rats (19). Furthermore, constitutively elevated plasma aldosterone levels are accompanied by an increased apical localization of ENaC in CNT but not CCD in mice lacking the thiazide-sensitive NaCl co-transporter (NCC) (20). Thus, the CCD may intervene only when the two preceding ASDN segments are overwhelmed (21). Even though late DCT and CNT, rather than CCD, are the main physiologic regulators of urinary Na\(^+\) and K\(^+\) excretion, many of the functional data in transgenic mice, such as electrophysiologic analysis in isolated perfused segments, are derived from CCD, mainly because of the technical difficulties to perfuse and study isolated late DCT and CNT.

**Gain-of-Function ENaC Mutants**

Liddle’s syndrome is a monogenetic form of arterial hypertension in which renal NaCl retention results from mutations and/or deletions of the PY motif in β- or γENaC. Ubiquitin ligases, such as Nedd4-2, interact with the PY motif, triggering internalization of ENaC from the luminal membrane and functional inactivation (Figure 1). Thus, endocytic retrieval of ENaC from the luminal membrane of the ASDN is thought to be reduced in Liddle’s syndrome resulting in a gain of function of ENaC (22). To elucidate the causal relationship among dietary salt intake, salt handling by the kidney, and hypertension, we generated a mouse model for Liddle’s syndrome using a knock-in strategy (Table 1) (23). These mice carry the β-ENaC-mutated allele with the R566stop mutation (Sensil\(^{b\text{Lid/Lid}}\)). It is interesting that the mice remain normotensive with a normal salt diet, despite evidence of hypervolemia and increased Na\(^+\) reabsorption in the large intestine. Moreover, plasma pH, Na\(^+\), K\(^+\), Cl\(^-\), or HCO\(_3^\) concentrations were not significantly affected under normal salt diet. However, the classical Liddle phenotype with higher BP, metabolic alkalosis, and hypokalemia accompanied by cardiac and renal hypertrophy manifests in the mice in response to a high-salt diet. The observed Na\(^+\) retention by the kidney of Liddle mice was linked to an increased density of conducting Na\(^+\) channels at the apical membrane of principal cells rather than to a change in open probability. Moreover, evidence for impaired ENaC internalization was demonstrated in vivo, as the increase in urinary Na\(^+\) excretion upon short time (6 to 12 h) salt repletion after 1 wk of low-salt diet is delayed in Liddle mice despite the presence of lower circulating aldosterone concentrations than in wild-type mice (24). In accordance, after 6 h of salt repletion, the γ-ENaC subunit is still retained at the apical cell membrane of CNT in Liddle mice but rapidly internalized in wild-type mice. At the same time, isolated perfused CCD from Liddle mice exhibit
higher transepithelial potential differences, and confluent primary cultures of CCD microdissected from their kidneys exhibit significant lower transepithelial electrical resistance and higher negative potential differences, all consistent with greater ENaC activity (24). Notably, mineralocorticoid upregulation of ENaC expression and function is still maintained in Liddle mice, which show a remarkable high sensitivity to aldosterone application in vivo (24,25). In a recent study, Chang and et al. (26) showed that renal CD cells from Liddle mice exhibit hyperactive apical vasopressin-regulated cystic fibrosis transmembrane conductance regulator (CFTR)–mediated Cl− conductance. Considering that hyperactive ENaC may establish a transepithelial potential difference that would be sufficiently negative to drive Cl− absorption, this effect could contribute to the enhanced NaCl reabsorption observed in the distal nephron of patients with Liddle’s syndrome. Finally, the observation that dysfunction of ENaC in the kidney of Liddle mice could be demonstrated before onset of arterial hypertension argues in favor of the kidney hypothesis proposed by Liddle et al. (27).

Loss-of-Function Mutants of the Mineralocorticoid Receptor

The mineralocorticoid receptor (MR) plays a central role for the genomic effects of aldosterone on kidney function. This is illustrated by studies in mice that lack MR. These mice die in the second week after birth, showing symptoms of PHA with hyponatremia, hyperkalemia, renal salt wasting, a strongly activated renin-angiotensin-aldosterone system, and a reduction of the amiloride-induced increase in fractional renal sodium excretion by 76% (28). Notably, no difference in amiloride-induced hyperpolarization of the basolateral membrane of isolated perfused medullary CD could be detected (29), indicating difficulties to pinpoint the proposed defect to the CD in these neonate mice. Further insights are provided by older mice as the mice can be rescued by daily matched NaCl substitution given from day 5 after birth (29). The NaCl-rescued 1-mo-old MR knockout mice display a fourfold greater fractional renal NaCl reabsorption observed in the distal nephron of patients with Liddle’s syndrome. Finally, the observation that dysfunction of ENaC in the kidney of Liddle mice could be demonstrated before onset of arterial hypertension argues in favor of the kidney hypothesis proposed by Liddle et al. (27).

MR-deficient mice demonstrate the importance of MR for ENaC-mediated renal Na+ reabsorption (Figure 1).

Loss-of-Function Mutants of Serum and Glucocorticoid Regulated Kinase 1, an Aldosterone-Induced ENaC Regulatory Protein

Aldosterone affects ENaC function in ASDN in part through serum and glucocorticoid regulated kinase 1 (Sgk1; for review, see 31,32). Aldosterone induces the expression of Sgk1, and activated Sgk1 upregulates Na+ channel activity at least in part by increasing ENaC protein abundance in the cell membrane. This interaction may involve phosphorylation and presumably thereby inactivation of the ubiquitin-ligase Nedd4-2 as well as more direct effects of the kinase on α-ENaC (33). Importantly, Sgk1 can also stimulate Na+/K+-ATPase; thus, Sgk1 may activate both apical and basolateral transport pathways required for transcellular Na+ reabsorption in the ASDN with Cl− following through paracellular pathways (Figure 1). Insights into the physiologic relevance of Sgk1 in kidney function was provided by studies in Sgk1 knockout mice. These mice present normal BP, GFR, and renal NaCl excretion when fed a normal NaCl diet. Evidence for a renal defect on standard NaCl diet, however, was provided by the observation that Sgk1 knockout mice display modestly higher plasma aldosterone and K+ concentrations (34). Restriction of dietary NaCl disclosed that the ability to upregulate renal NaCl reabsorption is significantly impaired in mice that lack Sgk1 despite the presence of excessive plasma aldosterone concentrations and decreased BP and GFR. Micropuncture experiments revealed compensatory proximal tubular hyperreabsorption and evidence for a Na+ transport defect in a segment including the early ASDN (34). Electrophysiologic analysis of isolated perfused CCD and immunohistochemistry of CNT demonstrated that the kinase is not absolutely necessary for the insertion of ENaC into the apical membrane and ENaC activation but that Sgk1 is required for the upregulation of apical membrane ENaC abundance and Na+ reabsorption in the ASDN in response to reduced NaCl intake (34).

Luminal ENaC and basolateral Na+/K+-ATPase are the two transport systems that establish the electrochemical basis for apical K+ secretion through the renal outer medullary K+ channel ROMK, which is of major importance for renal K+ excretion and body K+ homeostasis. Through its effects on these two transport systems, Sgk1 would be expected to influence renal K+ excretion (Figure 1). Furthermore, Sgk1 may upregulate ROMK1 activity also in a more direct way (for review, see 31,32). Plasma K+ concentrations under standard NaCl (and K+) diet were either not significantly different between genotypes (35) or modestly increased in Sgk1 knockout versus wild-type mice (4.7 versus 4.1 mM) (34). Thus, in the absence of Sgk1, modestly enhanced aldosterone release may serve to stabilize plasma K+ concentrations, reflecting an impaired renal ability to excrete K+. Indeed, we demonstrated an impaired upregulation of renal K+ excretion in Sgk1-deficient mice in response to both acute and chronic K+ loading. The
experiments revealed a defect in the K⁺ driving force, i.e., ENaC and/or Na⁺/K⁺-ATPase, rather than in ROMK expression and ROMK channel trafficking to the cell membrane as the dominant cause for the impaired upregulation: The absolute and the amiloride-sensitive transepithelial potential difference is reduced and cell membrane ROMK expression is actually increased in the ASDN of Sgk1 knockout mice as compared with wild-type mice in response to high K⁺ diet (35). The findings do not rule out an in vivo stimulating effect of Sgk1 on ROMK channel trafficking to the cell membrane because the lacking influence of Sgk1 could be more than compensated for by the observed enhanced plasma concentrations of K⁺ and/or aldosterone in Sgk1 knockout mice if these influences enhance apical ROMK abundance partially independent of Sgk1 (Figure 1). Figure 1 also illustrates a proposed parallelism with regard to the regulation of ENaC and ROMK by Sgk1 and protein kinase A, indicating the potential influence of vasopressin V₂ receptor activation. Mice that lack a functional V₂ receptor die within days after birth as a result of renal water loss associated with hypernatremia (36). ENaC activity has not been studied in these mice.

More recent studies demonstrated that the increase in NaCl appetite in response to the mineralocorticoid desoxycorticosterone acetate is attenuated in mice that lack Sgk1 (37), indicating that Sgk1 plays at least a dual role in mineralocorticoid-regulated NaCl homeostasis. Sgk1 dependence of both NaCl intake and renal NaCl reabsorption would make the kinase an attractive candidate gene for arterial hypertension with gain-of-function mutations stimulating NaCl intake in the presence of an impaired ability to excrete NaCl through the kidney. Indeed, Busjahn et al. (38) identified two polymorphisms of the Sgk1 gene that correlate with enhanced BP in twin studies. A gain-of-function Sgk1 mouse model could provide important further insights in this regard.

Perspectives

The outlined models show that genetically altered mice are valuable tools to validate and to define the importance of pathways/proteins that are implicated in ENaC-mediated Na⁺ transport and BP regulation. The same is likely to apply to other candidate pathways/proteins that have been identified but tested so far only in cell culture systems, such as the insulin pathway or genes such as Ned44/Ned44-like proteins, n-ncy downstream regulated gene 2 (39), or channel-activating proteases (CAP) (40,41). CAP are transmembrane-bound serine proteases that have been identified as positive regulators of ENaC. Thus, ENaC activity could be regulated by the activity of these serine proteases expressed at the surface of the same cell (Figure 1). This presents a new mechanism for autocrine activation of ion channels and may involve direct or indirect cleavage (40–42). We also found in vitro a synergistic activation of ENaC by CAP and Sgk1 that may allow a large dynamic range for ENaC-mediated Na⁺ regulation that is crucial for a tight control of Na⁺ homeostasis (42). Further roles of this serine protease have been proposed in Na⁺ absorption in kidney and lung (43). We recently generated a conditional allele at the mouse CAP1 (Prss8) gene locus that enables us specifically to ablate CAP1 function in the ASDN and other tissues (44). These transgenic mice should help to define the role of these genes in the kidney and other tissues/organs and serve as mammalian models of human diseases and later on to validate drug targets.

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