Glucocorticoid-induced leucine zipper protein regulates sodium and potassium balance in the distal nephron

Priyanka Rashmi[1], GianLuca Colussi[2], Michael Ng[1], Xinhao Wu[1], Atif Kidwai[1] and David Pearce[1]

[1]Division of Nephrology, Department of Medicine, University of California, San Francisco, California, USA; and [2]Department of Experimental and Clinical Medical Sciences, University of Udine, Udine, Italy

Glucocorticoid induced leucine zipper protein (GILZ) is an aldosterone-regulated protein that controls sodium transport in cultured kidney epithelial cells. Mice lacking GILZ have been reported previously to have electrolyte abnormalities. However, the mechanistic basis has not been explored. Here we provide evidence supporting a role for GILZ in modulating the balance of renal sodium and potassium excretion by regulating the sodium-chloride cotransporter (NCC) activity in the distal nephron. *Gilz*$^{-/-}$ mice have a higher plasma potassium concentration and lower fractional excretion of potassium than wild type mice. Furthermore, knockout mice are more sensitive to NCC inhibition by thiazides than are the wild type mice, and their phosphorylated NCC expression is higher. Despite increased NCC activity, knockout mice do not have higher blood pressure than wild type mice. However, during sodium deprivation, knockout mice come into sodium balance *more quickly*, than do the wild type, without a significant increase in plasma renin activity. Upon prolonged sodium restriction, knockout mice develop frank hyperkalemia. Finally, in HEK293T cells, exogenous GILZ inhibits NCC activity at least in part by inhibiting SPAK phosphorylation. Thus, GILZ promotes potassium secretion by inhibiting NCC and enhancing distal sodium delivery to the epithelial sodium channel. Additionally, *Gilz*$^{-/-}$ mice have features resembling familial hyperkalemic hypertension, a human disorder that manifests with hyperkalemia associated variably with hypertension.

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Correspondence: *David Pearce, University of California San Francisco, 600 16th Street, San Francisco, California 94143, USA. E-mail: David.Pearce@ucsf.edu*

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The kidney, in particular the aldosterone-sensitive distal nephron (ASDN), plays a critical role in regulating potassium (K^+) and sodium (Na^+) flux to achieve homeostasis of body fluid volume and plasma K^+ concentration.[1] Plasma K^+ is maintained within an extremely tight range in humans and mice.[2] Abnormalities in plasma K^+ can alter cellular electrical excitability with the potential of causing life-threatening cardiac arrhythmias.[3] On the other hand, the Na^+ balance of the body is critical for the maintenance of extracellular fluid volume and a normal blood pressure (BP).[4]

Aldosterone is a key hormone that orchestrates Na^+ and K^+ balance in response to environmental conditions, particularly changes in dietary Na^+ and K^+ availability, by inducing the expression of target genes such as glucocorticoid-induced leucine zipper protein (GILZ) and serum and glucocorticoid-regulated kinase 1 (SGK1). These gene products and their downstream signaling pathways further modulate the synthesis, trafficking, stability, and intrinsic activity of various ion transporters and channels.[5,6] In the distal convoluted tubule, Na^+ is reabsorbed predominantly by the electroneutral Na-Cl cotransporter (NCC) and in the connecting tubule and collecting duct by the electrogenic epithelial sodium channel (ENaC). ENaC-mediated sodium reabsorption depolarizes the apical membrane, generating a lumen negative voltage that provides the electrical driving force for K^+ secretion.[7] Recent studies have increasingly supported a role for the coordinated regulation of electroneutral and electrogenic Na^+ transport in the distal nephron in controlling Na^+ and K^+ balance.[8] Electroneutral reabsorption of Na^+ via NCC does not stimulate K^+ secretion, but in fact inhibits K^+ secretion by competing with ENaC-mediated Na^+ reabsorption.[9] During Na^+ depletion, aldosterone increases Na^+ reabsorption without stimulating K^+ secretion, and there is increasing evidence for a model in which the WNK (with no lysine = K) kinase network integrates systemic volume and K^+ status to fine-tune NCC, ENaC, and possibly renal outer medullary K channel (ROMK) activities.[8,10] It is postulated that a high concentration of circulating angiotensin II during Na^+ depletion activates the WNK1/4-SPAK (STE20/SPS1-related proline/alanine-rich kinase)-NCC pathway, thus favoring electroneutral Na^+ reabsorption.[11] It has been suggested that aldosterone also acts through the mineralocorticoid receptor and SGK1 to stimulate NCC.[12] However, this explanation cannot fully explain how plasma K^+ concentrations are maintained in the

context of prolonged sodium deficiency as hyperkalemia would eventually develop due to reduced Na^+ delivery to the ENaC-expressing nephron segments, resulting in reduced K^+ secretion. Therefore, understanding the intracellular signaling networks and identifying novel mediators that play a key role in striking an appropriate balance between electroneutral and electrogenic Na^+ transport in the distal renal tubule in response to systemic inputs including volume status, K^+ status, and aldosterone is critical.

GILZ is a member of the transforming growth factor β-stimulated clone 22 domain (TSC22D) family.[13] So far, 4 GILZ isoforms have been identified; but only isoform 1 (GILZ1) stimulates ENaC-mediated Na^+ current in $mpkCCD_{c14}$ cells and *Xenopus laevis* oocytes.[14] GILZ1 expression is strongly induced by aldosterone in kidney as well as in $mpkCCD_{c14}$ cells.[15] In *in vitro* models, GILZ1 physically interacts with and inhibits Raf-1, leading to ENaC activation.[16] GILZ1 also physically interacts with other components of ENaC regulatory complex, in particular SGK1.[17] GILZ1 increases SGK1 association with key substrates Nedd4-2 and Raf-1, as well as ENaC, while protecting SGK1 from rapid ER-associated proteasome-mediated degradation.[16,17] In spite of the extensive *in vitro* data, the role of GILZ in mediating ion transport *in vivo* remains unclear, and its role in NCC regulation, in particular, has not been examined.

As of now, we are aware of 3 distinct *Gilz* knockout ($Gilz^{-/-}$) mouse strains that have been characterized (in Suarez *et al.*,[18] Bruscoli *et al.*,[19,20] and the present work) and used to evaluate different GILZ functions. Based on *in vitro* studies, which showed that GILZ stimulates ENaC-mediated sodium current and modulates T-cell activation,[13,15,21] it was expected that $Gilz^{-/-}$ mice would exhibit signs of type 1 pseudohypoaldosteronism (PHA1) and defects in T-cell mediated immunity. Surprisingly, $Gilz^{-/-}$ males are sterile due to impaired spermatogenesis but display no tendency to Na^+ wasting and no obvious defects in immune function.[18,19] However, minor but consistent defects in Na^+ and K^+ handling have been reported.[18] The aim of the present study was to determine whether GILZ regulates whole-animal Na^+ and K^+ balance, and if so, to begin to assess the physiological conditions, the transporters involved, and the underlying signaling mechanisms. We have generated a whole-body $Gilz^{-/-}$ mouse and used a series of evocative tests to study the involvement of GILZ in ion transport regulation. Interestingly, unlike the PHA1 phenotype of SGK1-deficient animals, the phenotype of $Gilz^{-/-}$ mice resembles familial hyperkalemic hypertension/type 2 pseudohypoaldosteronism, resulting from hyperactivation of the WNK-SPAK-NCC pathway. Our data support the idea that a major role of GILZ is to inhibit WNK-SPAK–mediated activation of NCC, and thereby prevent the development of hyperkalemia in Na^+-avid states.

RESULTS

Generation of $Gilz^{-/-}$ mice

To address GILZ function *in vivo*, we generated mice constitutively deficient for all known TSC22D3-2 isoforms. The strategy for the generation of mice with a *Gilz* knockout allele is described in detail in Materials and Methods section. A previous study used a distinct but similar strategy to constitutively delete *TSC22D3-2*.[18] Consistent with their report, we found that $Gilz^{-/-}$ mice are viable, and born with normal body weight, but the males are sterile.[18,19] The defect in male fertility and failure of sperm maturation was found to be due to the loss of GILZ2 isoform,[19] whereas studies in *Xenopus laevis* oocytes and numerous cell lines support a role for GILZ1 in regulation of ENaC.[15] On a normal Na^+ diet (0.49% NaCl), there were no significant differences in the body weight (wild type 24.5 ± 0.6 g vs. knockout 24.4 ± 0.5 g, not significant); food intake (wild type 3.3 ± 0.1 g/day vs. knockout 3.2 ± 0.1 g/day, not significant); and water intake (wild type 3.8 ± 0.1 ml/day vs. knockout 3.8 ± 0.2 ml/day, not significant) between 10- to 12-week-old $Gilz^{+/+}$ and $Gilz^{-/-}$ mice.

Plasma electrolyte abnormalities in $Gilz^{-/-}$ mice

First, we examined electrolyte homeostasis and BP in the $Gilz^{-/-}$ mice. Comparison of plasma electrolyte levels in $Gilz^{-/-}$ mice with those of $Gilz^{+/+}$ littermates on a normal Na^+ diet uncovered notable differences in Na^+, K^+, and Cl^- concentrations. Plasma K^+ in $Gilz^{-/-}$ mice (4.9 ± 0.1 mM) was significantly higher than in $Gilz^{+/+}$ mice (4.3 ± 0.1 mM) (Figure 1a) consistent with a prior report.[18] In addition, $Gilz^{-/-}$ mice had significantly higher plasma Na^+ levels (Figure 1b). BP, however, was not significantly different between $Gilz^{+/+}$ and $Gilz^{-/-}$ mice (Table 1). Additionally, there was a modest but significant elevation in plasma Cl^- (Table 2).

Abnormal renal electrolyte handling in $Gilz^{-/-}$ mice

Urinary electrolytes showed that despite elevated plasma K^+, $Gilz^{-/-}$ mice showed no increase in urinary K^+ excretion, consistent with a renal defect in tubular K^+ secretion. This was reflected by a significantly lower fractional excretion of K^+ in $Gilz^{-/-}$ mice (Figure 1c and Table 3). Consistent with the findings of Suarez *et al.*,[18] we did not observe any obvious Na^+-excretion defect on a normal salt diet (0.49% NaCl) in $Gilz^{-/-}$ mice (Figure 1d and Table 3). Additionally, total chloride excretion was significantly reduced in $Gilz^{-/-}$ mice (Table 3). Taken together, the plasma and urinary electrolyte profiles of $Gilz^{-/-}$ mice closely resembled those found in familial hyperkalemic hypertension, and notably the $WNK4^{D561A/+}$ knock-in mouse model reported by Yang *et al.*[22] Hence, we considered the possibility that the WNK-SPAK-NCC pathway could be inappropriately upregulated in $Gilz^{-/-}$ mice.

NCC phosphorylation and functional activity are increased in $Gilz^{-/-}$ mice

In order to examine the activation state of NCC, we studied its expression and phosphorylation in membrane preparations from kidneys of $Gilz^{+/+}$ and $Gilz^{-/-}$ mice under basal conditions. Phosphorylation of NCC at key activating residues T53 and T58[23] was significantly higher in $Gilz^{-/-}$ mice (Figure 2). We did not detect any changes in total NCC; full