Hypokalemia in a Mouse Model of Gitelman Syndrome

by

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Running title: Potassium depletion in NCC (-/-) mice.

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ABSTRACT


We now show that modest reduction of dietary potassium induced a marked reduction in plasma potassium and elevated renal potassium excretion in NCC (-/-) mice, which was associated with a pronounced polydipsia and polyuria of central origin. These findings are consistent with the development of potassium depletion in NCC (-/-) mice and were not seen in wild-type mice maintained on the same low potassium diet. In addition, plasma aldosterone levels were significantly elevated in NCC (-/-) mice even in the presence of a low potassium diet. Collectively, these findings suggest an early central component to the polyuria of Gitelman syndrome and show that both elevated aldosterone and dietary potassium content contribute to the development of hypokalemia in Gitelman syndrome. Therefore, NCC (-/-) mice are more sensitive to reductions in dietary potassium than wild-type mice and become hypokalemic thus more faithfully representing the Gitelman phenotype seen in humans.

Index words: Potassium, Thiazide, Polyuria, Polydipsia, Aldosterone
INTRODUCTION

Hypokalemia is a cardinal feature of Gitelman’s syndrome, a genetic renal disorder also characterized by alkalosis, hypomagnesemia, hypocalciuria and mild salt wasting (6). Loss of function mutations in the thiazide-sensitive, sodium-chloride cotransporter (NCC) have been identified as underlying causes of Gitelman’s syndrome (23). Notably, administration of thiazide diuretics, agents widely used in the treatment of hypertension, generally results in the same physiological profile observed in Gitelman’s subjects, including potentially severe hypokalemia. Therefore, inhibition of NCC either through pharmacological means or as a result of genetic inactivation can result in clinically significant renal potassium loss.

A mouse model of Gitelman’s syndrome, NCC (-/-) mice, faithfully recapitulates many of the physiological findings observed in Gitelman’s patients including hypomagnesemia, hypocalciuria (13; 22), and alkalosis (13). Surprisingly, hypokalemia has not been detected in this model. Here we sought to determine if NCC (-/-) mice were more sensitive to reductions in dietary potassium intake than wild-type mice.

Hypokalemia is often induced in rodent models by severely restricting dietary potassium for a period of two weeks. In addition to reducing plasma potassium, prolonged potassium deprivation also results in polyuria, polydipsia, renal hypertrophy and impaired renal concentrating ability (18). Two weeks of potassium deprivation will induce the aforementioned changes in wild-type rodents with an intact NCC; therefore, we chose to shorten the duration and degree of potassium restriction in an effort to highlight any sensitivity of the NCC (-/-) mice relative to wild-type mice.
METHODS

Animals. A colony of NCC (-/-) mice on a C57/Bl6 background was established at the NIH from breeding pairs provided by Dr. Gary Shull, (University of Cincinnati; Cincinnati, Ohio). Control mice, wild-type C57/Bl6 whose age and sex were matched to experimental NCC (-/-) mice were purchased from Taconic and allowed to equilibrate for a minimum of three days in the NIH animal housing facility before experimentation. All studies were approved by the NHLBI ACUC.

Serum chemistries: Mice were anesthetized with isofluorane and blood collected by retro-orbital puncture using a glass Pasteur pipette. Whole blood was transferred to a serum separator (StatSpin Inc, Norwood, MA) and plasma was isolated by centrifugation. Electrolyte content of 150 ul of serum was analyzed at the Clinical Center Laboratory of Medicine.

Metabolic cage studies. Mice were housed in metabolic cages for durations ranging from 7 to 10 days. Mice were initially fed a standard rodent pellet food with ad lib water. Mice were then fed gel food diets containing 4.5g/25g BW of sodium and potassium free rodent meal supplemented with appropriate amounts of sodium (0.5 mEq/day) and potassium chloride, 3mls of water, and agar were used to control potassium intake. All mice, regardless of genotype, consumed essentially the entire daily portion of gel food. Our potassium replete diet contained 0.48 mEq of potassium chloride/day whereas the reduced potassium diet contained 0.048 mEq/day. In addition to the water present in the gel food, mice also had free access to water with the exception of the water restriction protocol. In the water restriction protocol, water intake was limited to 2.0mls of water/25g body weight in the gel food. Water intake was measured daily and mice were weighed at least every other day. Urine was collected under oil and urine volume and osmolality were determined gravimetrically and with a vapor pressure osmometer respectively. Electrolyte content of the urine was analyzed at the NHLBI Laboratory of Animal Medicine and Surgery.

Semi-quantitative immunoblotting: Mice were euthanized by cervical dislocation and
right kidneys processed as previously described (3). Equal loading was confirmed by staining gels as described previously (12). This gel was subsequently scanned with a linear fluorescence scanner (Odyssey, Li-Cor Biosciences) at an excitation wavelength of 700 nm. Affinity-purified primary antibodies against NKCC2, and AQP2 have been characterized previously.

**Aldosterone measurements:** Trunk blood was collected after decapitation and plasma collected via a serum separator. Plasma aldosterone was measured by radioimmunoassay (DPC; Los Angeles, California) per the manufacturer's instruction.

**Statistics.** ANOVAs were performed on multiple group comparisons followed by Bonferonni post-hoc tests. Significance was taken at $P<0.05$. Student’s T-tests were utilized on comparisons between two groups.
RESULTS

Serum Potassium. The plasma potassium concentration of NCC (-/-) mice on a low potassium diet was significantly decreased (P < 0.01) by roughly 1 mM compared to all other groups (Figure 1). As previously documented (22) NCC (-/-) mice displayed hypomagnesemia, and this was exacerbated by a low potassium diet (Figure 1). In contrast, plasma sodium and calcium concentrations were not significantly different between groups (data not shown).

Urinary Potassium Excretion. Rates of urinary potassium excretion were measured in NCC (-/-) and wild-type mice. After initiation of a low potassium diet, NCC (-/-) mice had significantly higher rates of renal potassium excretion compared to wild-type mice (Figure 2). Over time, the difference in urinary potassium excretion between NCC (-/-) and wild-type mice became indistinguishable, establishing a new steady state at a lower absolute rate of potassium excretion.

Time Course of Water Intake, Urine volume and Urine Osmolality. Polydipsia and polyuria are well known consequences of hypokalemia (18). A representative time course of water intake (panel A), urine output (panel B), and urine osmolality (panel C) of wild-type and NCC (-/-) mice are presented in figure 3. In the basal condition, days 1-3, there was no significant difference in water intake, urine output, or urine osmolality between wild-type or NCC (-/-) mice. After two days on a reduced potassium diet (day 5 fig 3), there was a significant increase in the water intake of the NCC (-/-) compared to all other groups (fig 3 panel A). This increase in water intake in the NCC (-/-) mice became increasingly pronounced over the duration of the experiment and was not observed in the other groups of animals. Significant increases in urine volume (panel B) and significant reductions in urine osmolality (panel C) of the NCC (-/-) low potassium group paralleled the increases in water intake. Among four metabolic cage studies performed, the onset of polyuria and polydipsia in the NCC (-/-) mice varied from two to four days after introduction of the low potassium diet but always persisted through the duration of the experiment.
**Urinary Concentrating Ability.** A urinary concentrating test was performed on all four groups of mice to investigate if the pronounced polyuria and polydipsia observed in the NCC (-/-) mice on a reduced potassium diet were associated with a reduced urine concentrating ability. Water intake, urine osmolality and urine volumes before and after 24 hours of water restriction for all four experimental groups are presented in figure 4. All groups of animals significantly increased urine osmolality and decreased urine volume in response to water restriction. Urine osmolality and urine volume of wild-type and NCC (-/-) groups on low potassium diets were not significantly different after 24 hrs of water restriction.

**Kidney Weight.** Kidney weights and kidney weights normalized for body weight were analyzed for evidence of renal hypertrophy. Two of four individual experiments demonstrated a significant increase in kidney weight in NCC (-/-) mice maintained on a low potassium diet compared to knockout mice on a normal potassium diet (i.e. 0.116 ± 0.004 g vs. 0.149 ± 0.008 g N=10 P < 0.002). However, cumulative results from the 4 experiments (30-40 mice from each experimental group) did not demonstrate significant differences in kidney weight or kidney weight normalized to body weight despite the consistent polydipsia and polyuria (Data not shown).

**Western blotting analysis.** Alterations in the expression pattern of transport proteins involved in renal concentrating ability were analyzed by Western blotting of whole kidney homogenates prepared from mice with ad libitum access to water. The protein abundance of the water channel aquaporin 2 (AQP2) was significantly reduced in both wild-type and NCC (-/-) mice on low potassium diets. In contrast, the abundance of NKCC2 was not significantly different among the groups (Figure 5).

**Plasma Aldosterone.** Plasma aldosterone measurements were performed on the four experimental groups (Figure 6). Plasma aldosterone was significantly higher (P < 0.001) in NCC (-/-) mice compared to wild-type mice regardless of diet. As expected, low potassium diets...
suppressed plasma aldosterone levels although statistical significance was achieved only for
the NCC (-/-) mice (P < 0.01). Even on a low potassium diet, aldosterone levels in NCC (-/-)
mice were markedly elevated at 2.2 ± 0.5 nM (compared with the Kd of the mineralocorticoid
receptor for aldosterone: 1.3 nM).
**Discussion**  Here we have examined the effects of manipulating dietary potassium intake in a mouse model of Gitelman’s syndrome. In response to a diminished potassium intake, NCC (-/-) mice demonstrated a pronounced polydipsia and polyuria concurrent with elevated renal potassium excretion and significantly reduced serum potassium and magnesium concentrations; findings consistent with the development of potassium depletion. These findings were not observed in wild-type mice on a reduced potassium diet or wild-type and NCC (-/-) mice on a diet replete with potassium. Thus, the NCC (-/-) mice are more sensitive to dietary potassium restriction than wild-type mice.

In contrast to the seven day potassium depletion studies presented here, many experimental protocols for potassium depletion are two weeks in duration (1,2,9,15) and encompass both central and renal responses. For example, Berl et al. (1), have demonstrated polydipsia preceeds, and is independent of, the ultimate appearance of urinary concentrating defects in potassium depletion. Our results demonstrated the early onset of polydipsia and polyuria in the NCC (-/-) mice coincident with significant renal potassium loss. In NCC (-/-) mice, the polydipsia and polyuria persisted throughout the duration of the experiment despite wild-type and NCC (-/-) mice ultimately achieving similar rates of renal potassium excretion. The potassium loss accrued in the NCC (-/-) mice before establishing the new steady-state level of potassium excretion resulted in significantly lower plasma potassium in the NCC (-/-) mice which persisted throughout the duration of the experiments. The persistent polydipsia and polyuria is thought to be driven, at least in part, by the effects of hypokalemia on neural regulation of the thirst drive. However, many compensatory changes with various time courses are undoubtedly occurring in response to potassium depletion, including possible changes in the vasopressin axis, and angiotensin II levels which may directly or indirectly contribute to the polydipsia. Nevertheless, the absence of a frank renal concentrating defect at the specific time point examined in this model is consistent with an early-onset primary polydipsia. Such a process undoubtedly contributes to the polyuria seen in Gitelman patients.
Renal hypertrophy and a urinary concentrating defect are normally considered hallmarks of potassium depletion induced by restricting potassium intake. However, despite the obvious polydipsia, polyuria and significantly reduced plasma potassium levels, at this time point there was no evidence of a concentrating defect or consistent findings of renal hypertrophy in the NCC (-/-) mice maintained on a low potassium diet. We propose that the lack of a urinary concentrating defect and inconsistent findings of hypertrophy are a result of the relatively short duration of low potassium diet which is further aggravated by the variable onset of polydipsia and polyuria.

The absence of a renal concentrating defect and inconsistent signs of renal hypertrophy would at first appear to preclude a renal phenotype at this specific time point in the model. However, there were significant decreases in renal AQP2 transporter abundance in both wild-type and NCC (-/-) mice in response to a low potassium diet. The diminished expression of AQP2 would be expected to contribute to the subsequent development of a renal concentrating defect known to be present after two weeks of potassium depletion (1,7,17). Furthermore, the decreases in AQP2 abundance seen in wild-type and NCC (-/-) mice on low potassium diets are independent of water intake since water intake is significantly elevated only in the NCC (-/-) low potassium group, while AQP2 levels are decreased in both. Again, these findings are consistent with the observations that the ultimate development of a concentrating defect in potassium depletion is independent of water intake (1) and imply the kidneys are responding to dietary potassium through an unknown mechanism.

Given the exacerbated hypomagnesemia observed in these studies, the potential role of magnesium depletion in this model should be considered. Magnesium depletion is known to cause tissue potassium depletion (24) but does not result in a urinary concentrating defect (16). However, in models of primary magnesium depletion, there is no evidence of polyuria or polydipsia (16). Furthermore, the hypomagnesemia described in this study is enhanced only in response to reductions in dietary potassium intake. Therefore, the exacerbated
hypomagnesemia would appear to be secondary to alterations in potassium metabolism and not directly involved the polydipsia and polyuria.

There are many potential renal mechanisms contributing to the potassium deficiency in NCC (-/-) mice on a low potassium diet. First, even on a low potassium diet, circulating aldosterone in the NCC (-/-) mice is markedly elevated (presumably due to the salt wasting) and this would serve as a potent stimulus for potassium secretion by the aldosterone-sensitive segments of the renal tubule (4; 19). The hypocalciuria previously reported in the NCC (-/-) mice may also play an indirect role in promoting potassium secretion. Urinary calcium has been shown to inhibit active potassium secretion (20); therefore, hypocalciuria could relieve this inhibition and promote potassium secretion. Furthermore, calcium also inhibits the activity of ENaC (5; 8). Again, a reduction of this inhibitory influence would allow enhanced electrogenic sodium reabsorption resulting in a depolarization of the apical membrane of the collecting duct principal cells, thereby increasing the driving force for potassium secretion (21). Finally, increased luminal flow, as might be encountered in this model, has been shown to stimulate potassium secretion in in-vivo-perfused cortical collecting ducts (14). Further investigation will be required to identify the precise molecular mechanisms in this model.

The pathogenesis of hypokalemia in Gitelman's syndrome remains incompletely understood (10). Most explanations favor one or more of the aforementioned mechanisms. We have established that in a mouse model of Gitelman's syndrome a modest reduction in dietary potassium intake can induce the hypokalemic phenotype, allowing more detailed investigation of mechanisms contributing to the development of hypokalemia. Previous studies have shown that there are significant fluctuations in dietary potassium intake in humans, and that these are associated with blood pressure changes and cardiovascular risk (11). Conceivably, hypokalemia in Gitelman's syndrome may be associated with a low dietary potassium intake, and may explain why some patients develop hypokalemia and polyuric spells, whereas others patients remain normokalemic.
In conclusion, NCC (-/-) mice are sensitive to reductions in dietary potassium and begin to demonstrate signs of potassium depletion before wild-type mice. Our findings are consistent with previous studies demonstrating polyuria and polydipsia occurring early in the development of potassium depletion, with alterations in renal concentrating ability and morphology occurring at later time points. Thus the absence of hypokalemia in NCC (-/-) mice on a normal diet appears to due to compensation by sufficient amounts of dietary potassium.

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Figure Legends.

Figure 1. Plasma potassium and magnesium concentrations of wild-type and NCC (-/-) mice after 7 days on normal and low potassium diets. * denotes P < 0.05 by ANOVA.

Figure 2. Urinary excretion of potassium in wild-type and NCC (-/-) mice maintained on low potassium diets. * denotes P < 0.05 by Student’s T-test.

Figure 3. Time course of average water intake (panel A), urine output (panel B), and urine osmolality (panel C) of wild-type and NCC (-/-) mice under basal conditions (Day 1-3, Green numbering) and upon switching to gel-food diets containing normal and low potassium (Day 4-10).

Figure 4. Urinary concentrating ability of wild-type and NCC (-/-) mice maintained on normal and low potassium diets.

Figure 5. Western blot of whole kidney homogenates demonstrating the effects of manipulating dietary potassium on NKCC2 and AQP2 expression in wild-type and NCC (-/-) mice. * denotes P < 0.05 by ANOVA.

Figure 6. Plasma aldosterone levels of wild-type and NCC (-/-) mice maintained on normal or low potassium diets for 7 days. * denotes P < 0.05 by ANOVA.
Reference List


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