FEMALE ROMK NULL MICE MANIFEST MORE SEVERE BARTTER II PHENOTYPE ON
RENAL FUNCTION AND HIGHER PGE₂ PRODUCTION

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ABSTRACT

ROMK knockout null mice with a high survival rate and varying severity of hydronephrosis provide a good model to study type II Bartter syndrome pathophysiology (26). During the development of such a colony we found that more male than female null mice survived, 58.70% vs. 33.33%. To investigate the possible mechanism of this difference, we compared the survival rates, renal functions, degree of hydronephrosis as well as PGE$_2$ and TXB$_2$ production between male and female ROMK wild-type and null mice. We observed that female ROMK Bartter’s mice exhibited lower GFR (0.37 vs. 0.54 ml/min/100gBW, P <0.05) and higher fractional Na$^+$ excretion (0.66% vs. 0.48%, P<0.05) than male Bartter’s. No significant differences in acid-base parameters, urinary K$^+$ excretion and plasma electrolyte concentrations were observed between genders. In addition, we assessed the liquid retention rate in the kidney to evaluate the extent of hydronephrosis and observed that 67% of male and 90% of female ROMK null mice were hydronephrotic mice. Urinary PGE$_2$ excretion was higher in both genders of ROMK null mice: 1.35 vs. 1.10 ng/24hr in males and 2.90 vs. 0.87 ng/24hr in females. TXB$_2$ excretion was higher in female mice in both wild-type and ROMK null mice. The increments of urinary PGE$_2$ and TXB$_2$ were significantly higher in female null mice than males, 233.33% vs. 22.74% of PGE$_2$ and 85.67% vs. 20.36% of TXB$_2$. These data demonstrate a more severe Bartter phenotype in female ROMK null mice, and higher PGE$_2$ and TXB$_2$ production may be one of the mechanisms of this manifestation.
INTRODUCTION

Bartter’s syndrome belongs to a group of rare autosomal-recessive disorders, characterized by salt wasting, polyuria, hypokalemic alkalosis with dehydration and increased renin and aldosterone levels (2, 39), caused by severe reductions of salt absorption along the thick ascending limb of Henle (TAL) (13). Bartter’s syndrome (28, 36) results from mutations in one of five proteins that are linked to loss of salt transport in the TAL (13). Type II Bartter’s syndrome is caused by mutations of the KCNJ1 gene on chromosome 11q24-25, encoding the inward-rectifier potassium channel ROMK (Kir1.1) (13, 41). One of the important physiological functions of ROMK channel is the generation of lumen-positive transepithelial potential, which drives the paracellular reabsorption of NaCl and K+, and also provides the apical pathway for K+ recycling. Such K+ recycling is critical for supporting Na⁺/2Cl⁻/K⁺-cotransport activity (45). ROMK knockout mice exhibit similar spectrums as Bartter’s syndrome in patients (24). The significant reduction of Cl⁻ absorption along the loop of Henle has been demonstrated in ROMK knockout mice (24). We have also demonstrated that ROMK protein expression in TAL and cortical collecting duct CCD and apical small-conductance K⁺ channel activity was absent in apical membranes from either TAL or CCD in knockout mice (26), and that these mice exhibited characteristics of Bartter’s syndrome including polyuria and Na⁺ and K⁺ wasting with compensatory increased water and food intake (26). The first study from ROMK null mice reported by Lorenz and Shull shows that the mortality of ROMK null mice was very high, and less than 5% survived to weaning at 21 days; the survival rate did not improve significantly by daily subcutaneous injections of either indomethacin or isotonic saline (24). However, the survival rate significantly increased by selectively crossing surviving ROMK null mutants with heterozygotes from litters in which there were surviving null mutants (25, 26). Since more male
than female null mice survived, 58.70% vs. 33.33%, during the development of a higher survival colony, we further investigated the gender difference in renal functions by both metabolic cage and renal clearance methods. The survival rates and degree of hydronephrosis in male and female ROMK null mice were also compared. We also examined urinary concentrations of both PGE2 and TXB2 in wild-type and ROMK null mice and compared the differences between genders. Our data show that female mice exhibit a more severe Bartter’s phenotype with a lower GFR, more severe hydronephrosis, higher PGE2 and TXB2 excretion, and earlier death.
METHODS

Animal Care, Reproduction and Growth:

The ROMK wild-type and knockout mouse colonies were generated as described previously (26). All mice were housed at the Yale animal facility with a temperature- and humidity-controlled environment and 12:12hr light-dark cycle. To produce ROMK null mice, four cross combinations were designed in order to confirm that the ROMK genotype was controlled by one recessive gene segregation pattern. These four combinations are normal heterozygous (female) X heterozygous (male), heterozygous (female) X homozygous (male), homozygous (female) X heterozygous (male), homozygous (female) X homozygous (male). The protocol used for genotyping pups was described previously and was followed in current experiments (26). After genotyping, mice were separated into four groups; wild-type male and female groups, and knockout male and female groups. The body weight was taken every week from birth to 6 weeks, and recorded monthly until the 22nd month.

Metabolic experiments:

The metabolic cage experiments were performed by methods described previously in our laboratory (26). Briefly, two mice (6 months old) were placed in a metabolic cage (Lab Products Inc, Seaford, DE) and trained for 2 days with free drinking water and standard pellet rodent chow. After training, mice that exhibited normal eating and drinking behavior were used for the experiments. Two mice from the same group (same sex, genotype and age) were housed in a single cage and the 24-hour urine volume, food and water intake were measured and recorded for 3 days. Urinary Na\(^+\) and K\(^+\) concentrations were measured by a flame photometer (IL 943 Automatic Flame Photometer).
Renal clearance and blood gas measurements:

Renal clearance experiments were carried out by following the methods previously developed in our laboratory (43). The mice were anesthetized by IP Inactin (100 to 150 mg/kg body weight) and placed on a thermostatically controlled surgical table, maintaining body temperature at 37°C. A tracheostomy was performed, the left jugular vein exposed and cannulated for intravenous infusion, and a carotid artery catheterized for arterial blood collection. The bladder was catheterized via a suprapubic incision for timed urine collections.

After surgical preparation, 0.05 ml of isotonic saline was given intravenously to replace surgical fluid loss. Subsequently, a priming dose of 10 µCi of \([\text{methoxy-}^3\text{H}]\) inulin (New England Nuclear, Boston, MA) was administered in 0.3-ml isotonic saline, and a maintenance infusion of Ringer solution containing 10 µCi/ml of inulin, at a rate of 0.41 ml/h. An equilibration period of 60 min was followed by 30 min collection periods. Arterial blood samples were taken in the middle of each urine collection period, and urine and plasma Na\(^+\) and K\(^+\) concentrations were measured by standard flame photometry. Absolute (\(E_{Na}, E_K\)) and fractional (\(F_{E_{Na}}, F_{E_K}\)) excretion rates were calculated by standard methods (42). The blood for pH, PCO\(_2\), and HCO\(_3^−\) measurements was collected by retroorbital bleeding and measured with a blood-gas analyzer (Blood-gas Corning 850, Corning Medical and Scientific).

Evaluation of hydronephrosis:

Kidney hydronephrosis was evaluated by the Liquid Retention Rate (LRR) at aged (12 ~18 month) wild-type and ROMK null mice. The liquid retention was examined by transecting the kidney in the coronal plane and draining retained liquid in the kidney with Kimwipes paper. Kidneys were weighed before and after cutting and draining, and the ratio of liquid weight and fresh kidney weight were calculated as LRR. Two methods were used to assess hydronephrosis:
one is average LRR in which two kidneys from each mouse were calculated; another is hydronephrosis scales in which only the kidney with the higher LRR was used. Based on maximum LRR of wild-type mice and 75 % percentile of LRR in knockout mice, we rated three hydronephrosis scales: normal, slight and severe hydronephrosis. Due to LRR variation in two kidneys of same animal, each mouse’s LRR was represented by the higher LRR kidney to evaluate hydronephrosis scale in knockout mouse.

**Measurement of urinary PGE$_2$ and TXB$_2$**:

*Urine collection:* Mice were housed in clear metabolic cages with free access to normal food and water. Urine samples were collected in tubes over 16 h. The collecting tubes were immersed in ice during collection.

*PGE$_2$ measurement:* PGE$_2$ concentration was determined by using a PGE$_2$ EIA Kit-Monoclonal (catalog No. 514010, Cayman Chemical, Ann Arbor, MI). The assay was performed according to the manufacturer's instructions. Briefly, after diluting samples to the optional concentration, 50 µl of each sample along with a serial dilution of PGE$_2$ standard samples were mixed with appropriate amounts of acetylcholinesterase-labeled tracer and PGE$_2$ antiserum, and incubated at 4°C for 18 h. After the wells were emptied and rinsed with wash buffer, 200 µl of Ellman's reagent containing substrate for acetylcholinesterase was added. The enzyme reaction was carried out on a slow shaker at room temperature for 1-2 hr. The plates were read with Microplate Reader (Benchmark Microplate Reader, BIO-RAD) at 405 nm. The results were analyzed by Cayman Chemical’s computer spreadsheet.
Thromboxane B₂ (TXB₂) measurement: TXB₂ concentration was determined by using a Thromboxane B₂ EIA Kit (catalog No. 519031, Cayman Chemical, Ann Arbor, MI). The assay was very similar to the PGE₂ measurement. Briefly, 50 µl of each diluted sample (1:10) along with a serial dilution of TXB₂ standard samples were added to the plate well, followed by 50 ul TXB₂ AChE tracer and TXB₂ antiserum, and the plate was incubated at room temperature for 18 h. After the wells were emptied and rinsed with wash buffer 5 times, 200 µl of Ellman's reagent containing substrate for acetylcholinesterase was added to each well and developed on a slow shaker at room temperature for 1 hr. Methods to read the plate and calculate the result were same as above in PGE₂ measurement.

Statistic analysis

Data are presented as the mean ± SE. Statistical significance was assessed by using paired and unpaired t test depending on whether paired or unpaired experiments were conducted. Pearson’s correlation analysis was used for testing correlations between PGE2, TXB2 excretion and LRR.
RESULTS

Sex ratio and survival rate in male and female ROMK Bartter’s mice:

The sex ratio of newborn pups and their survival rates were examined in male and female ROMK knockout mice. The results are summarized in Fig. 1. The study included 138 male and 141 female newborns. The ratio of male and female is close to 1:1, indicating no differences of reproductive and embryonic development between genders in ROMK null mice. Since most of non-surviving ROMK knockout mice died between birth and 15 days (26) and death was rare after weaning for up to six months, the survival rates were compared at the weaning stage of development (3 weeks old). The average survival rates were calculated from animals produced from different cross combinations, +/- X +/-, +/- X -/-, -/- X +/- and -/- X -/-. As shown in Fig. 1 the male knockout pups had significantly higher survival rates than female pups. The rates are 57% in males and 34% in females respectively.

Growth trends in male and female ROMK Bartter’s mice:

The growth trends were evaluated by measuring age-dependent changes of body weight in ROMK wild-type and null male and female mice. Body weights at birth, and their weekly changes before weaning in all types of mice are summarized in Fig. 2, showing significantly lower weights from both genders of ROMK null mice at almost all age periods. The lower body weight in ROMK null mice is consistent with slower development and growth in children with Bartter’s syndrome (30, 36).

Figure 3 summarizes the body weight changes recorded for a time period of two years in all types of mice. Based on the general changes of body weight, mouse growth can roughly be divided into three periods; namely the developmental, growth continuation and stabilization of body weight periods. Figure 3 shows that the body weight quickly increased in the first 6 months
then slowed from 7 to 14 months; after 15 months, the body weight remained relatively constant. These three periods of body weight changes are clearly demonstrated in both male and female wild-type mice, although the overall body weights are significantly lower in female than male mice (Fig. 3). In ROMK knockout mice, mean body weights are lower in both male and female mice, with no significant differences in the rate of increase in body weight in the developmental period. However, after 7 months the body weight becomes constant without significant change, and the continuation growth period is absent in the ROMK null mice. The difference between male and female KO mice also appeared after 14 months. In that period, the body weight was constant in males but significantly reduced in female KO mice, suggesting the possibility of progressive renal functional impairment in female mice. Indeed, aged females exhibited higher death rates than males (17.5% vs. 10.53%) when evaluated for the period of 15 to 26 months.

**Comparison of 24-hour urine output, Na⁺ and K⁺ excretion:**

Urine output, total Na⁺ and K⁺ excretion, and water and food intake were examined in conscious mice by metabolic cage experiments. Data are summarized in Table 1 and show significantly higher urine volume and Na⁺ and K⁺ excretion in both genders of ROMK null mice, consistent with salt and water wasting seen in Bartter’s patients and previously reported in ROMK null mice (24, 26). Food and water intake in ROMK null mice were also significantly higher than in wild-type mice of both genders. Such increase of water and food intake is expected and compensates for the salt and water wasting in ROMK Bartter’s mice. There were no significant differences in urine output, absolute Na⁺ and K⁺ excretion between genders either in wild-type mice or in ROMK null mice. However, food and water intake were significantly higher in female than male ROMK null mice, indicating more water wasting may occur in female null mice.
Comparison of GFR, urine volume, Na\(^+\) and K\(^+\) excretion:

Urine volume, GFR, and fractional Na\(^+\) and K\(^+\) excretion were examined by renal clearance methods in all groups of anesthetized mice. The results are summarized in Table 3. Urine volumes were much higher in both genders in ROMK-null mice, consistent with the polyuria in Bartter’s syndrome. In addition to the lower GFR in ROMK-null mice compared to wild-type mice of both genders, the female ROMK-null mice had an even lower GFR than males. This result demonstrates more severely impaired renal functions in female ROMK-null mice.

No differences in fractional Na\(^+\) and K\(^+\) excretions were observed between genders in wild-type mice (Table 3). FE\(_{Na}\) was significantly elevated in knockout mice of both genders, consistent with sodium wasting in Bartter’s syndrome. FE\(_{Na}\) was higher in females than male knockout mice, indicating more severe sodium wasting in female null mice. FE\(_{K}\) is also higher in knockout mice in both genders. However, there was no statistically significant gender difference in FE\(_{K}\) in female null mice. Plasma electrolyte and acid-base parameter measurements were summarized in Table 2 and shown no significant difference in Na\(^+\), K\(^+\) and Cl\(^-\) concentrations among all groups except for male knockout mice which statistically lower than male wild-type ones. In addition, blood pH and HCO\(_3^-\) had no significant difference between genotypes in each gender. The blood pH and HCO\(_3^-\) levels are slightly lower than those measured from arterial blood (44), since the mixed blood might be obtained by retro-orbital bleeding in this study. As we reported previously (26), the hematocrit was higher in ROMK null mice, suggesting that extracellular fluid is reduced in null mice.

Comparison of LRR to Evaluate Hydronephrosis:

Since no methods were available to determine the presence of, or to quantify the severity of, hydronephrosis in mice, we examined whether the amount of liquid retention in the kidney
would provide a reasonable index to hydronephrosis. This seemed reasonable since hydronephrosis would reduce renal mass and increase kidney fluid retention in a dilated, hydrenephrotic collecting system. Thus, a higher LRR than normal would consider hydronephrosis. Fig. 4 showed the LRR in male and female wild-type and ROMK null mice kidney. There was no difference between genders in wild-type mice. In ROMK null mice, the average LRR was higher in female than male: 17.75 ± 2.46% (n=36, from 18 mice) in male and 21.44 ± 2.31% (n=40, from 20 mice) in female. Because of the large variation of LRR from each kidney, this difference was not statistically significant. Further, we determined the normal range of LRR values and the 75% percentile LRR in wild-type mice. The LRRs were low and ranged between 2.6 ~ 8.9% and the 75% percentile LRR was 5.2% from 24 kidneys in adult wild-type mice aged 12~18 months. Neither LRR ranges nor the 75% percentile LRRs showed difference between genders or between two kidneys from individual wild-type mice. Thus we used these normal values in wild-type mice to indicate the absence of hydronephrosis.

In 76 kidneys from ROMK null mice aged 12~18 months, LRR range was larger (1.4 ~ 65%) and the 75% percentile LRR was higher (25.8%) than in wild-type mice. Based on the finding that LRRs are less than but close to 10% in all wild-type mice and the 75% percentile LRR in ROMK null mouse kidneys is about 25%, we used 10 and 25% as thresholds to rate normal (LRR ≤10%), slight hydronephrosis (10< LRR ≤25%) and severe hydronephrosis (LRR>25%) mice. Fig. 5 showed the percentages of knockout mice with normal, slight and severe hydronephrosis in both genders. The results indicated that 33, 27 and 40% males and 10, 40 and 50% females exhibited normal, slight and severe hydronephrosis, respectively. 33% of males, but only 10% of females were without hydronephrosis, and 67% of male and 90% female
were hydronephrotic, indicating that female ROMK mice had more significant hydronephrosis than males.

**Comparison of PGE₂ and TXB₂ production:**

The urinary PGE₂ amount was compared in wild-type and ROMK null mice in both genders, as shown in figure 6A. In wild-type mice, the PGE₂ production was slightly lower in female than male, 0.87±0.06 (n=6) vs. 1.10 ±0.1 ng /24hr (n= 12), P=0.1. In contrast, female null mice had significantly higher PGE2 excretion (2.90 ±0.61 vs. 1.35±0.19 ng /24hr, n=13, 25 respectively, P=0.0043) compared with wild-type, consistent with high prostaglandin excretion in the urine in Bartter’s syndrome (7, 9). The increments of PGE₂ excretion in ROMK null mice over wild-type mice were 233.33% in female and only 22.73% in male. Fig. 6B shows the urinary TXB₂ excretion in wild-type and ROMK null mice in both genders. The female wild-type mice exhibited higher urinary TXB₂ production than males, 3.21 ± 0.31 vs. 1.67 ± 0.21 ng/24hr (n=8 for both genders, P=0.0011). The TXB₂ excretion increased by 20.36% from 1.67 ± 0.21 to 2.01 ± 0.31 ng/24hr (n=8, 20, respectively, P=0.41) in male ROMK null mice. Meanwhile, the TXB₂ increased by 85.67% from 3.21 ± 0.31 to 5.96 ± 0.62 ng/24hr in female ROMK null mice (n=8, 14, respectively, P<0.0045). The difference in TXB₂ excretion between male and female ROMK null mice was extremely significant.

To investigate whether severe hydronephrosis is correlated to increased PGE₂ or TXB₂ production, we compared the LRR, PGE2 and TXB2 concentrations between male and female ROMK null mice (Fig. 7). The correlation analysis was performed, and the significance of the curves was calculated as compared to correlation coefficients at zero. Fig. 7A shows the relationship of hydronephrosis evaluated by LRR and urinary PGE₂ excretion in male and female ROMK null mice. Despite the increase in LRR from 10 to 50%, PGE₂ excretions were at a
similar level in male ROMK null mice (Fig. 7A). The slope of the first order regression curve was -0.01984, and the correlation coefficient (Pearson’s r) was -0.22381, P=0.43. In contrast, PGE₂ excretions were higher in female ROMK null mice as the LRR was elevated. The slope was 0.1036, Pearson’s r was 0.9147, P=0.0039. Fig. 7B displays the relationship of hydronephrosis and urinary TXB₂ excretion in male and female ROMK null mice. Urinary TXB₂ excretion was much higher at almost all points in female than male null mice. TXB₂ excretions failed to exhibited correlation with LRR in male mice; however, LRR and TXB₂ excretion seemed to be negatively correlated in female null mice. The slope was -0.081, the Pearson’s r was -0.7966 and P=0.032 (Fig. 7B).
DISCUSSION

Mice lacking ROMK exhibit a phenotype similar to Bartter’s syndrome, including salt and water wasting, and high PGE₂ excretion (9, 33). This study shows that female ROMK Bartter’s mice exhibit a greater reduction in GFR, a greater magnitude of hydronephrosis, higher urinary PGE₂ and TXB₂ excretion rates, and reduced growth and survival rates. Although the mechanism of these gender differences is not yet clear, one possible contributing factor is the elevated PGE₂ production in female null mice. PGE₂ has been found to inhibit distal nephron NaCl absorption (15, 16), and the inhibition of PGs production by indomethacin and COX-2 inhibitors has been used as the major method to treat Bartter’s patients (11, 31). Such treatment not only prevented salt wasting and dehydration, but also slowed the process of progressive renal failure, especially in Bartter’s children (40).

Three lines of evidence indicate that female ROMK null mice exhibit more severe and progressive impairment of renal function than males. First, female mice have lower rates of GFR and elevated fractional sodium excretion; second, females had more severe hydronephrosis; and third, females had a greater reduction of body weight and a lower survival rate. It is likely that the gender differences in magnitude of body weight reduction and earlier death in female ROMK null adults was due to the greater impairment of renal function with hydronephrosis. The suggestive evidence supports the theory that increased PGE₂ production in female ROMK null mice may be one of the important contributors to the severity of the Bartter’s phenotype in this gender. COX-2 is a major enzyme in the kidney for PGE₂ synthesis and its expression increases in high-renin states (12). Higher COX-2 expression has also been detected in Bartter’s patients (21, 31). The role of PGE₂ in Bartter’s syndrome is supported by the observations that inhibition of PGs generation with indomethacin and COX-2 inhibitors reduces the salt wasting
dehydration and hypercalciuria in children with hyperprostaglandin E syndrome (6, 18). Previous studies show that the nonselective COX inhibitor indomethacin causes sodium retention and a decline in GFR, but the selective inhibition of COX-2 does not, suggesting that the depression of GFR by indomethacin is due to inhibition of Cox-1 (4). The effect of PGE$_2$ on Na$^+$ transport in the kidney has been extensively studied and it has been demonstrated that PGE$_2$ inhibits net Na$^+$ absorption in the CCD and inner medullary collecting duct (IMCD) (3), and reduces apical Na$^+$ channel activity in rabbit cortical collecting tubules (23). PGE$_2$ also reduces Cl$^-$ absorption in the loop of Henle (29), inhibits Na-K-2Cl cotransport activity in medullary thick ascending limb cells (19), and reduces IMCD Na$^+$-K$^+$-ATPase activity (16). Given the condition of salt wasting and dehydration, inhibition of Na$^+$ absorption by PGE$_2$ would enhance the severity of salt wasting and diuresis in Bartter’s syndrome, an outcome that likely contributes to the incidence and magnitude of hydronephrosis in ROMK-null mice.

Previous studies reported that both PGE$_2$ and TXB$_2$ production increased in a hydronephrosis model induced by unilateral ureteral obstruction in rat (20), by renal vein constriction in rabbit (34) and in human congenital obstructive uropathy (22). These studies indicated that PGE$_2$ and TXB$_2$ are important pathophysiological regulators in response to increased hydronic pressure and to reduced renal blood flow. However, it is not clear whether higher PGE$_2$ and TXB$_2$ is responsible for severe hydronephrosis in female mice, or whether the increased PGE$_2$ and TXB$_2$ level is secondarily induced by hydronephrosis. To investigate this mechanism, we first examined urinary concentrations of both PGE$_2$ and TXB$_2$ in wild-type and ROMK null mice and compared the differences between genders. Our data show that both PGE$_2$ and TXB$_2$ excretion were increased significantly more in female than in male ROMK null mice (Fig. 8). Second, we compared the LRR, PGE$_2$ and TXB$_2$ concentrations between male and
female ROMK null mice. Our data show that the PGE$_2$ concentrations were higher and more correlated to the level of LRR in female compared to male ROMK null mice. In contrast, although the urinary TXB$_2$ excretion was much higher in female than male null mice TXB$_2$ excretions failed to exhibited correlation with LRR in male mice; and the LRR and TXB$_2$ excretion seemed to be negatively correlated in female null mice. It should be noticed that more numbers of animals are needed to measure PGE2, TXB2 and hydronephrosis, especially in animals with higher levels of LRR as shown in Fig. 7. However, a view of a lower survival rate of female knockout mice and a very small percentage of knockout mice having 60% of LRR (Fig. 4), from present data, we are not able to conclude that PGE2 is directly causing the hydronephrosis.

The reasons for significantly higher production/excretion of PGE$_2$ in female ROMK null mice are still under investigation. However, it has been found that estrogen increases PGE$_2$ concentrations and AT1 receptor expression in the renal cortex and medulla in mice (1). Estrogen also up-regulates PGE$_2$ synthesis and down-regulates PGE$_2$ degradation by inhibiting 15-PGDH activity (10). It has also been established that Ang II stimulates PGE$_2$ (14, 37) and TXB$_2$ excretion (46) and the renin-Ang II system is elevated in Bartter’s syndrome (21). Given the fact that both Ang II and estrogen increase the level of PGE$_2$, the additive effect of Ang II and estrogen on PGE$_2$ production could result in more severe renal functional deficiencies in female than in male Bartter’s. Studies of gender differences in spontaneously hypertensive rats demonstrated that PGE$_2$ and TXB$_2$ respond to sex hormones differently (38). These studies found enhanced urinary excretion of PGE$_2$ and TXB$_2$ with increased COX-2 expression in female compared with male SHR. Orchidectomy was associated with increased PGE$_2$ metabolite excretion and microsomal PGE synthase protein expression suggesting that testosterone inhibits
PGE₂ synthesis, but TXB₂ excretion was not affected by gonadectomy in either male or female SHR (38).

Several lines of evidence from previous studies supported the hypothesis that increased PGE₂ in ROMK null mice is induced by higher renin-angiotensin system (RAS) activity. First, studies in rats with chronic Ang II infusion increased PGE₂, PGI₂ production and also COX-2 expression at the mRNA and protein levels in isolated glomeruli and in mesangial cells (17). Secondly, chronic administration of furosemide, which causes effects similar to Bartter’s syndrome, increased both plasma Ang II and urinary PGE₂ excretion. A high correlation between the increments in Ang II and urinary PGE₂ was found and the increase in PGE₂ was blocked by Captopril (8). However, it has also been reported that Ang II inhibits COX-2 expression and angiotensin converting enzyme inhibitor increases COX-2 mRNA and renal cortical COX-2 immunoreactivity in the macula densa, and Agtr1a and Agtr1b null mice show increased COX-2 expression in the cTAL and the macula densa (5). The relative role of Ang II in regulation of COX-2 in glomeruli, mesangial cells and in the macula densa and cTAL needs to be investigated.

The mechanism of the hydronephrosis in ROMK null mice is not clear since higher urine volume should not increase the hydronic pressure in the kidney unless there is obstruction of urine output downstream of the kidney. In our renal clearance experiments with 40 plus ROMK null mice of both genders, we observed that bladders of knockout mice were always full of urine before the cannulation, and the sizes of the bladders were estimated to be 2 to 3 times larger than those in WT mice. Although we were unable to measure the pressure in the bladder, it is reasonable to assume that the pressure was higher, since the bladder muscle wall always looks very thin in the ROMK null mice compared to WT. Such large amounts of urine accumulated in
the bladder may increase the renal hydronic pressure, which in turn may result in hydronephrosis. Why large amounts of urine accumulate in the bladder of ROMK null mouse, and whether the knockout of the ROMK channel alters the $K_{ATP}$ channel activity in the bladder, needs to be investigated.

PERSPECTIVES AND SIGNIFICANCE

Previously, most observations in experimental animals and in humans (including ageing, diabetes and polycystic kidney disease, acute and chronic ischemic renal failure), have shown that female and/or supplementary treatments with estradiol were found to attenuate the progression of renal disease (27, 32, 35). However, we have found in ROMK Bartter’s mouse models that female ROMK Bartter’s mice exhibit a lower GFR, a more severe hydronephrosis, a higher fractional Na$^+$ excretion, and a greater reduction in growth and survival rates. We have suggested that elevated PGE$_2$ and TXB$_2$ production in female ROMK null mice may be one of the contributing factors for the more severe phenotype in this gender. It remains unclear, however, why higher PGE$_2$ and TXB$_2$ are exhibited and how they contribute to accelerated renal failure in female ROMK null mice.

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References:


Figure legends:

Fig. 1 The survival rates in male and female ROMK null mice from birth to weaning. The percentage of survival was analyzed by the ratio of the animal numbers at birth (day 1) and the number of survivors at subsequent days.

Fig. 2 Comparison of body weight of ROMK wild-type and null pups before weaning. The body weights were measured and compared at birth (day 0), 1 week, 2 weeks and at weaning (3 weeks), respectively. *: P< 0.05, ** P<0.01.

Fig. 3 Growth trends of male and female ROMK wild-type and null mice. Four curves represent body weights from weaning to 22 months in male WT, female WT, male null and female null mice respectively. ROMK null mice had lower body weights than those in wild-type mice in both genders from 6-14 months of age. In addition, after 14 months of age, there was a significant reduction of body weight in female null mice compared to males.

Fig. 4. Liquid retention rates (LRRs) in male and female wild-type (WT-M, WT-F, n=12 for both groups) and ROMK knockout (KO-M, KO-F, n=36, 40, respectively) mice kidneys. Each symbol indicates the ratio of liquid and kidney weight.

Fig. 5. Hydronephrosis development in male and female ROMK knockout mice. Hydronephrosis of each mouse was scaled based on the liquid retention rate (LRR) in kidney. The mice with LRR ≤10% were classified as normal, 10<LRR≤25% as slight hydronephrosis, LRR>25% as severe hydronephrosis. N =18 for male, 20 female.

Fig. 6. Urinary PGE2 and TXB2 excretion in male and female wild-type and ROMK null mice. A. PGE2 excretions were significantly higher in female ROMK null mice but not in male null mice. *: Significant difference compared with the wild-type mice (P< 0.05);
#: Significant difference compared with the gender (P<0.05).

B. TXB₂ excretions were significantly higher in female than male in both wild-type and ROMK null mice. The increment of urinary TXB₂ excretion was significantly higher in female than in male null mice. *and # represent same as (A); ** and ## : P<0.01.

Fig. 7. A. The relationship of hydronephrosis (LRR) and PGE₂ excretion in male and female ROMK null mice. In females, PGE₂ concentrations are higher than male ROMK null mice and exhibited a positive correlation to LRR. The correlation coefficients (Pearson’s r) were 0.9147 (P=0.0039) in females and -0.2381 (P=0.4335) in male mice.

B. The relationship of LRR and TXB₂ excretion in male and female ROMK null mice. Females have higher TXB₂ excretion than male mice. There was no correlation between LRR and TXB₂ excretion in male null mice (Pearson’s r =-0.1992, P=0.5141), but showed a negatively correlation in female ROMK null mice (Pearson’s r = -0.7966, p=0.032).
Table 1. Comparison of food and water intake, urine output, sodium and potassium excretion between male and female ROMK wild-type and null mice.

<table>
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<tr>
<th>Sex</th>
<th>N</th>
<th>BW (g)</th>
<th>Food intake (g/24 hr/10g BW)</th>
<th>Water intake (ml/24 hr/10g BW)</th>
<th>UV (ml/24 hr/10g BW)</th>
<th>ENa (uEq/24 hr/10g BW)</th>
<th>EK (uEq/24 hr/10g BW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>M</td>
<td>20</td>
<td>27.60±0.65</td>
<td>0.82±0.07</td>
<td>1.05±0.12</td>
<td>0.36±0.05</td>
<td>33.7±3.85</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>18</td>
<td>24.50±0.57+</td>
<td>1.03±0.10</td>
<td>1.03±0.07</td>
<td>0.38±0.04</td>
<td>39.6±4.18</td>
</tr>
<tr>
<td>KO</td>
<td>M</td>
<td>26</td>
<td>25.96±0.72</td>
<td>1.18±0.08*</td>
<td>3.36±0.22*</td>
<td>2.21±0.13*</td>
<td>57.1±4.65*</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>10</td>
<td>22.60±1.17+</td>
<td>1.53±0.16*+</td>
<td>4.43±0.37*+</td>
<td>2.34±0.16*</td>
<td>58.8±6.91*</td>
</tr>
</tbody>
</table>

N: number of animals; BW: body weight; UV: Urine volume; ENa and EK: Absolute Na\(^+\) and K\(^+\) excretion.
* Significant difference between wild-type and knockout mice in same gender
+ Significant difference between genders in same genotype

Table 2. Plasma electrolyte and acid-base parameters in male, female ROMK null and wild-type mice.

<table>
<thead>
<tr>
<th>Sex</th>
<th>N</th>
<th>PNa (mM)</th>
<th>PK (mM)</th>
<th>PCI (mM)</th>
<th>pH</th>
<th>PHCO(_3)^- (mM)</th>
<th>HCT(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>M</td>
<td>151.8±2.5</td>
<td>4.32±0.20</td>
<td>112.8±2.0</td>
<td>7.32±0.03</td>
<td>21.9±0.8</td>
<td>47.8±0.6</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>149.7±1.4</td>
<td>4.37±0.26</td>
<td>112.4±1.0</td>
<td>7.25±0.01</td>
<td>20.9±0.8</td>
<td>44.6±1.34</td>
</tr>
<tr>
<td>KO</td>
<td>M</td>
<td>155.5±1.1</td>
<td>3.68±0.16*</td>
<td>113.8±2.4</td>
<td>7.27±0.02</td>
<td>22.9±0.7</td>
<td>49.3±1.8</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>157.6±2.8</td>
<td>3.97±0.08</td>
<td>115.3±3.0</td>
<td>7.23±0.04</td>
<td>21.9±1.1</td>
<td>49.3±1.3*</td>
</tr>
</tbody>
</table>

N: number of animals; PNa, PK, PCI: plasma Na\(^+\), K\(^+\) and Cl\(^-\) concentrations; HCT: hematocrit; * Significant difference from same gender wild-type (P<0.05).
Table 3. Urine volume, GFR, Na$^+$ and K$^+$ excretion in male, female ROMK null and wild-type.

<table>
<thead>
<tr>
<th>Sex</th>
<th>N</th>
<th>UV (ul/min/100g BW)</th>
<th>GFR (ml/min/100g BW)</th>
<th>ENa (uEq/min/100g BW)</th>
<th>EK (uEq/min/100g BW)</th>
<th>FENa (%)</th>
<th>FEK (%)</th>
<th>MBP (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>M</td>
<td>8</td>
<td>2.55±0.45</td>
<td>0.84±0.06</td>
<td>0.39±0.04</td>
<td>0.39±0.08</td>
<td>0.33±0.05</td>
<td>20.00±2.69</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>8</td>
<td>3.15±0.24</td>
<td>0.71±0.13</td>
<td>0.33±0.07</td>
<td>0.44±0.08</td>
<td>0.32±0.03</td>
<td>17.92±1.27</td>
</tr>
<tr>
<td>KO</td>
<td>M</td>
<td>9</td>
<td>7.66±0.93***</td>
<td>0.54±0.04*</td>
<td>0.42±0.06</td>
<td>0.79±0.07*</td>
<td>0.48±0.04*</td>
<td>43.58±2.52*</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>8</td>
<td>10.1±1.25***</td>
<td>0.37±0.05*+</td>
<td>0.34±0.06</td>
<td>0.58±0.08</td>
<td>0.66±0.09*+</td>
<td>42.88±8.89*</td>
</tr>
</tbody>
</table>

N: number of animals; UV: urine volume, ENa, EK: Absolute Na$^+$ and K$^+$ excretion; FENa, FEK: Fractional Na$^+$ and K$^+$ excretion; MBP: Mean blood pressure.

* Significant difference between wild-type and knockout mice (*: P<0.05; ***: P<0.001).
+ Significant difference between genders in same genotype (+: P<0.05).
Fig. 1.
Fig. 2

Body Weight (g)

- M-WT
- M-KO
- F-WT
- F-KO

0 day 1wk 2wk Weaning

*Statistical significance
**Stronger statistical significance
Fig. 3
Fig. 4.

Liquid retention in kidney (%)
Male Female

N = 18, 20 respectively

Age range: 12-18 months

\[ \text{LRR: Liquid Retention Rate} \]

Severe: LRR >25%
Slight: LRR 10-25%
Normal: LRR <10%

\[ \text{N = 18, 20 respectively} \]

Age range: 12-18 months

Fig. 5
<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TXB</strong> (_2) (ng/24/hr) N=8 N=20</td>
<td><img src="image" alt="Bar chart" /></td>
<td><img src="image" alt="Bar chart" /></td>
</tr>
<tr>
<td><strong>PGE</strong> (_2) (ng/24/hr) N=12 N=25</td>
<td><img src="image" alt="Bar chart" /></td>
<td><img src="image" alt="Bar chart" /></td>
</tr>
</tbody>
</table>

Fig. 6.
Fig. 7.