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MOLECULAR AND CELLULAR MECHANISMS OF DISEASE

H,K-ATPase type 2 contributes to salt-sensitive hypertension induced by K$^+$ restriction

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Abstract In industrialized countries, a large part of the population is daily exposed to low K$^+$ intake, a situation correlated with the development of salt-sensitive hypertension. Among many processes, adaptation to K$^+$-restriction involves the stimulation of H,K-ATPase type 2 (HKA2) in the kidney and colon and, in this study, we have investigated whether HKA2 also contributes to the determination of blood pressure (BP). By using wild-type (WT) and HKA2-null mice (HKA2 KO), we showed that after 4 days of K$^+$ restriction, WT remain normokalemic and normotensive (112 ± 3 mmHg) whereas HKA2 KO mice exhibit hypokalemia and hypotension (104 ± 2 mmHg). The decrease of BP in HKA2 KO is due to the absence of NaCl-cotransporter (NCC) stimulation, leading to renal loss of salt and decreased extracellular volume (by 20 %). These effects are likely related to the renal resistance to vasopressin observed in HKA2 KO that may be explained, in part by the increased production of prostaglandin E2 (PGE2). In WT, the stimulation of NCC induced by K$^+$-restriction is responsible for the

elevation in BP when salt intake increases, an effect blunted in HKA2-null mice. The presence of an activated HKA2 is therefore required to limit the decrease in plasma [K$^+$] but also contributes to the development of salt-sensitive hypertension.

Keywords Blood pressure · Extracellular volume · Low-K$^+$ diet · Vasopressin · Na$^+$/Cl$^-$-cotransporter

Introduction

Disturbances in the potassium balance are at the origin of many complications and are potentially life-threatening. The organism is rather well protected against hyperkalemia; however, when it occurs, its consequences (which are mainly cardiac) are rapidly fatal. Hypokalemia is a disorder more frequent than hyperkalemia. The prevalence of hospitalized patients with a plasma [K$^+$] < 3 mM is 3–5 % [29, 30] and reaches 12 % when the cut-off is 3.5 mM [9]. Its origins are generally multifactorial and involve vomiting, diarrhea, diuretic abuse and can be linked also to genetic disorders (Conn's disease, Bartter and Gitelman syndrome, for instance). In addition to pathological states leading to hypokalemia, dietary behaviors may also contribute to lower the whole body K$^+$ content. In developed Western countries, consumption of Na$^+$ has increased well above physiological needs whereas that of K$^+$ has dramatically decreased [11]. Studies focusing on the impact of a low daily intake of K$^+$ in humans have demonstrated a positive correlation with the probability for developing hypertension by mechanisms that remain partly unsettled [7, 14, 19]. Conversely, analysis of K$^+$-depleted animal models (normal or hypertensive) suggests that a low K$^+$ intake induces a decrease in blood pressure (BP) [18, 21, 28]. This discrepancy is probably not related to species

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differences in the blood pressure response to dietary K^+ restriction but is rather due to the degree of K^+ restriction, which is moderate in human studies and almost complete in animal studies but also to the large amount of Na^+ normally ingested by the general population compared to the experimental models. Recent studies [3, 37, 39, 40] highlight the role of the thiazide-sensitive Na^+/Cl^--cotransporter (NCC) in the development of a salt-sensitive hypertension induced by a low-K^+ diet. However, the molecular trigger of NCC stimulation in this context is still debated since it could be hypokalemia [37] or peptide hormones like vasopressin (AVP), for instance [3].

Many coordinated regulatory processes are involved in regulating the potassium balance [42]. In the case of dietary K^+ restriction, the retention of K^+ is favored, via the inhibition of renal K^+ secretion through the K^+ channel ROMK (for review see [41]) and the stimulation of K^+ reabsorption through a progesterone-dependent activation of the H,K-ATPase type 2 (for review see [8]). This last process has been challenged by the lack of renal phenotype in HKA2-null mice fed a low-K^+ diet [22]. More recently, however, we have discovered that HKA2-null mice do not retain K^+ correctly during gestation [34] and exhibit circadian defects in urinary excretion of K^+ that affects plasma K^+ levels [33]. Moreover, when we acutely inhibited the K^+-restriction-induced stimulation of HKA2 by blocking the nuclear progesterone receptor, the mice displayed a higher loss of K^+ in their urine and a lower plasma K^+ level [12]. These recent data showed that the renal expression of HKA2 is not an epiphenomenon and that HKA2-null mice, as many knock-out models, may develop compensatory systems to override the gene deletion.

Since HKA2 contributes to the regulation of the K^+ balance and modifications of the dietary K^+ intake alters BP levels, we aimed at better determining whether HKA2 helps in controlling BP under K^+-depletion conditions.

Material and Methods

Physiological analysis

Experiments were performed on C57Bl6 wild-type (WT) and knock-out mice for the HKA2 α subunit gene [22]. All the animals were house kept at CEF (Centre d'Explorations Fonctionnelles of CRC, Agreement no. A75–06-12). They were maintained in a constant temperature and humidity in light controlled room with a 12 h light cycle. They had free access to food (SAFE Laboratory) and tap water. All experiments were conducted in accordance with the institutional guidelines and the recommendations for the care and use of laboratory animals put forward by the Directive 2010/63/EU revising Directive 86/609/EEC on the protection of animals used for scientific purposes (project has been approved by a user establishment's ethics committee and the Project

Authorization: number 2289.01). To record physiological parameters, mice were placed in metabolic cages (Techniplast, France) and were fed either a standard laboratory diet (0.28 % Na^+ and 0.6 % K^+; Safe France) or a low-K^+ diet (0.28 % Na^+ and 0.01 % K^+; Safe France). Urinary creatinine concentrations were determined using an automatic analyzer (Konelab 20i; Thermo, France). Urinary Na^+ and K^+ concentration was determined by flame photometry (IL943, Instruments laboratory, France). Urinary aldosterone content was determined using an EIA kit (USCN Life Science Inc) according to supplier's recommendation. Urine PGE2 was measured by using an EIA method according to supplier's information (Prostaglandin E metabolite EIA kit, Cayman Chemicals). Thiazide and amiloride-sensitive natriuresis were measured as previously described [20, 25]. To test for the vasopressin response, dDAVP (1.5 µg/kg in 100 µl of 0.9 % NaCl) or its vehicle were injected intraperitoneally and a spot of urine was taken 5 h post-injection for measurement of osmolality (Vogel 6300 Voebling osmometer, Bioblock Scientific) and compared to the osmolality of urine taken before injections. A specific V2 receptor antagonist (SR121463A, from Sanofi-Aventis, kindly provided by Nadine Bouby, Cordelier Research Center, INSERM [32]) was mixed in the food to provide 1.5 mg/animal. This treatment triggers a comparable polydipsia in WT and HKA2-null mice (40–44 ml/day in both strain). Plasma volume was determined by Evans blue dye dilution as previously described [2, 6, 24] in anesthetized animals (a mix of xylazine, 10 mg/kg and ketamine 100 mg/kg). Blood pressure was measured in conscious restrained mice by a tail-cuffed plethysmography method (BP2000, Visitech system, France) after a week of adaptation to the apparatus between 9:00 and 11:00 AM.

Quantitative PCR

RNAs were extracted from whole kidneys using the TRI reagent (Invitrogen, France) following the manufacturer's instructions. One µg of total RNA was then reverse-transcribed (Roche Diagnostics, France) and real-time PCRs were performed on a LightCycler (Roche Diagnostics, France) as previously described [15].

Membrane protein extraction and western blot analysis

Total kidneys were homogenized in a lysis buffer (250 mM sucrose, 100 mM Tris-Hepes, pH 7.4 and protease inhibitor cocktail (Complete, Roche Diagnostics)). After removal of aggregates and nuclear-associated membrane by low-speed centrifugations, the plasma membrane enriched fraction (17,000 × g for 30 min) was recovered into the lysis buffer and its protein content was measured with the BCA method (Thermo Scientific Pierce). Forty µg of protein were then denaturated, resolved by SDS-PAGE (10 % polyacrylamide)

and transferred onto a nitrocellulose membrane. Ponceau red labeling was carried out to check for protein loading accuracy. Western blots were performed according to the standard procedure using a polyclonal anti-total NCC antibody, an anti-ENaC γ subunit antibody kindly provided by Jan. Loffing (University of Zurich, [36]) or a goat-anti-AQP2 (Santa Cruz). For quantification, the band intensities were normalized by Ponceau red intensity rather than using a protein marker such as β-actin, the protein expression of which is affected by K^+ restriction [5].

Immunofluorescence on kidney slice

Before or after 4 days of K^+ restriction, anesthetized mice (10 mg/kg xylazine and 100 mg/kg ketamine) were perfused with 4 % paraformaldehyde in the aorta and the kidneys were removed and frozen in OCT. Five μm thick slices were then processed for immunofluorescence microscopy using an anti-AQP2 antibody (1/200) and confocal microscopy analysis (Leica LSM 710).

Data and statistical analysis

Results are expressed as mean ± s.e.m. The numbers of mice used in these experiments are indicated in the legends (n). One-way or two-way ANOVA tests as well as non-paired Student t-test were used to determine statistical significance (see the legends), differences with $p < 0.05$ were considered significant.

Results

Under K^+ restriction, the absence of H,K-ATPase type 2 accelerates the development of hypotension

As already observed [22, 33], the dietary K^+ restriction induced profound physiological changes (Table 1) that differ in their kinetics between WT and HKA2-null mice, including parallel loss of weight and food intake. HKA2-null mice have been shown to loose K^+ in their feces [22, 33, 34] thus accelerating the development of hypokalemia that is already present after

only 4 days of K^+ restriction whereas WT mice remained normokalemic (3.16 ± 0.11 vs 3.60 ± 0.09 mM, $p = 0.005$).

Systolic BP was measured in wild-type animals under normal K^+ conditions (NK, white bars) or under dietary K^+ restriction (LK, gray bars). It remained stable for up to 13 days under normal condition but start decreasing significantly after 13 days of K^+ restriction ($p < 0.01$ compared to day1 and to day13 of NK; Fig. 1A). Comparison between WT (white bars) and HKA2-null mice under normal dietary conditions (day −1 and day −2) shows no significant differences in their BP level (Fig. 1B). After a switch to a K^+ restricted diet, the HKA2-null mice decreased their BP (at d1-d2) than in WT mice. The difference in BP between the two genotypes reached 8–15 mmHg up to the 6th day of K^+ restriction. After that, the difference disappeared and both genotypes reached a similar, low BP at day 13 (around 100 mmHg).

Renal Na^+ and water losses are at the origin of the K^+-depletion induced hypovolemia in HKA2-null mice

We then directly measured plasma volume by injecting a known quantity of Evans blue dye in the circulation. These experiments were carried-out at day 4 of the low-K^+ diet, when BP has started to decrease in HKA2-null mice but remains normal in WT mice. As shown in Fig. 2A, the volume of the plasma compartment was similar before and after the low-K^+ diet switch in WT mice (934 ± 57 μl and 929 ± 50 μl, respectively). In contrast, in HKA2-null mice (Fig. 2B), plasma volume was 200 μl lower on the 4th day of the low-K^+ diet than before the switch (837 ± 49 μl and 1044 ± 67 μl, respectively; $p = 0.027$). As expected, WT mice under low-K^+ diet rapidly decreased their production of aldosterone (by 70 % compared to day-1, $p < 0.01$, Fig. 2C). In HKA2-null mice the decrease of urinary aldosterone is more modest and reach only 40 % compared to day-1 (p = 0.02 at day1). Thus, after 4 days of K^+ restriction, HKA2-null mice excreted 2 times more aldosterone than WT ($p < 0.01$), which may outline that the volume depletion observed in HKA2-null mice "antagonized" the effect of the K^+ depletion.

A 20 % decrease in plasma volume is likely to originate from a reduced ability of the kidney to retain Na^+ and water.

Table 1 Metabolic parameters of WT ($n = 15$) and HKA2-null mice ($n = 16$) under normal (day-1) and low K^+ diet (days 4 and 13). Results are shown as mean ± s.e.m. HKA2-null mice vs WT, non-paired Student t-test (* p < 0.05)

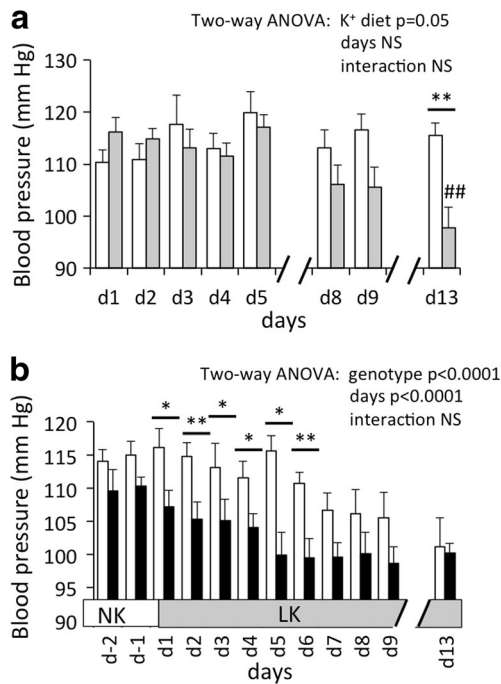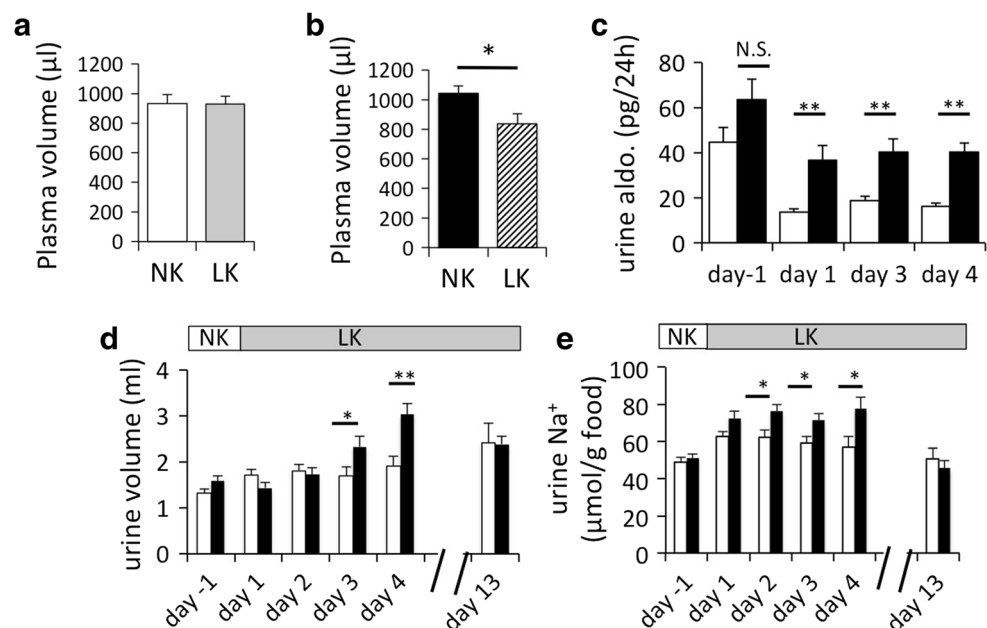| K^+ restriction (day)	Day −1		Day 4		Day 13	
	WT	HKA2-KO	WT	HKA2-KO	WT	HKA2-KO
Weight (g)	26.0 ± 1.0	26.3 ± 0.5	22.0 ± 1.4	22.6 ± 0.6	23.3 ± 0.9	$19.0 \pm 0.6^*$
Food intake (g)	4.3 ± 0.1	4.3 ± 0.2	3.1 ± 0.2	2.5 ± 0.2	3.7 ± 0.3	$2.4 \pm 0.3^*$
Plasma K^+ value (mM)	3.95 ± 0.10	4.12 ± 0.16	3.60 ± 0.09	$3.16 \pm 0.11^*$	3.26 ± 0.16	$2.80 \pm 0.13^*$
Urine K^+ μmol/24 h	515 ± 32	503 ± 27	12 ± 1	10 ± 4	12 ± 2	6 ± 1

Fig. 1 *Effect of dietary K^+ restriction on blood pressure (BP) level.* (A) Systolic BP of wild-type (WT) mice under normal (white bars) or low-K^+ diet (gray bars). Results are shown as the mean ± s.e.m. (n = 6–9). Two-way ANOVA test was performed to analyze the influence of diet (normal or low-K^+ diets) and diet duration on blood pressure. (B) Systolic BP in WT (white bars, $n = 15$–20 for period d-2 to d4 and n = 6–9 for d5 to d13) and HKA2-null mice (black bars, n = 15–20 for period d-2 to d4 and n = 6–8 for d5 to d13) before (d-2, d-1) and after a switch to a low-K^+ diet (d1 to d13). Results are shown as the mean ± s.e.m. Non-paired Student t-test (** $p < 0.01$; *$p < 0.05$ and ## $p < 0.01$ compared to d1). Two-way ANOVA test was performed to analyze the influence of genotype and diet duration on BP

We therefore measured urinary water and Na^+ excretion before and during a four-day period of low-K^+ diet. As shown in Fig. 2D urine volume was slightly increased (by 30 % at day4 compared to day-1, $p < 0.01$) in WT mice under a low-K^+ diet (white bars) during this period of 4 days and is almost doubled after 13 days of treatment (p < 0.01). In HKA2-null mice, urine volume increased faster than in in WT mice, with an increase of 50 % and 90 % at day 3 and 4 compared to day-1 (p < 0.01). Therefore, in the short period of K^+ restriction, the absence of HKA2 induced a higher loss of fluid than in WT mice (30 % at day3, $p = 0.042$ and 50 % at day4, p < 0.01). After a prolonged period of K+ restriction, WT and HKA2-null mice displayed a similar loss of fluid. Regarding Na^+ excretion (Fig. 2E), it was normalized by the amount of food intake so as to avoid biases stemming from the decrease in food intake occurring in both strains after the switch (as discussed above). In WT mice, urinary Na^+ excretion increased significantly but transiently in the first days after the switch to a K^+-restricted diet compared to NK diet (64 ± 3 μmol/g, $p < 0.01$; 62 ± 4 μmol/g, p < 0.01 and 59 ± 4 μmol/g, $p = 0.03$ at day 1, 2 and 3, respectively; vs. 49 ± 3 μmol/g under normal conditions) and returned to a normal value at day 4 (57 ± 6 μmol/g, $p = 0.165$) up to day 13 (50.8 ± 5.6 μmol/g, $p = 0.83$). In HKA2-null mice, urinary Na^+ excretion also increased significantly in response to K^+ restriction compared to normal diet (p < 0.01), with a stronger amplitude than that observed in WT mice since these mice lost around 10–20 μmol/g each day during the short period of K^+ restriction ($p = 0.05$ at day1, p = 0.03 at day2, $p = 0.02$ at day3 and 4). After a prolonged period of K^+ restriction, HKA2-null mice returned to a normal value (45.7 ± 4.3 μmol/g).

Fig. 2 *Volume of the plasma compartment and renal water and Na^+ excretion under low K^+ diet during K^+-depletion.* Plasma volume of WT (A, n = 15) and HKA2-null mice (B, n = 15) in normal conditions (NK) and after 4 days of a low-K^+ diet (LK). (C) 24 h urine excretion of aldosterone in WT (white bars, $n = 10$–12) and HKA2-null mice (black bars, n = 10–12) in normal conditions (day-1) and after a switch to a low-K^+ diet (day1 – day4). Urine volume (D) and Na^+ content (normalized to food intake, E) from WT (n = 10–12, white bars) and HKA2-null mice (n = 10–12, black bars) before (day-1) and after a low-K^+ diet (day 1-day 13). Results are shown as the mean ± s.e.m. Non-paired Student t-test (** p < 0.01; * $p < 0.05$)

Differential regulation of NCC and ENaC in WT and HKA2-null mice during early K⁺ restriction

Measurements of total NCC protein expression confirmed that it is increased under the low-K⁺ diet in WT (by 70 %, $p < 0.01$) but not in HKA2-null animals (Fig. 3A). Finally, the functional activity of NCC was assessed in vivo in both strains before and after a 4-day period of low-K⁺ diet, by measuring the acute natriuretic effect of a single dose of hydrochlorothiazide (ip injection of HCTZ, 50 mg/kg for 6 h). As shown in Fig. 3B, under normal-K⁺ conditions, HCTZ induced a similar natriuretic response in WT and HKA2-null mice (around 100 μmol Na⁺/μmol of creatinine). During K⁺ restriction, the natriuretic effect of HCTZ was increased by 50 % in WT mice (compared to normal K⁺ condition, $p < 0.01$). In HKA2-null mice, the response to HCTZ (122 μmol Na⁺/μmol creatinine) was not significantly increased compared to that under a normal K⁺ diet ($p = 0.097$) and was much weaker than that in WT ($p < 0.01$). Thus, NCC stimulation appears to be blunted in HKA2-null mice after 4 days of K⁺ restriction.

The protein expression of the γ subunit of ENaC (90 kDa band) is decreased by 25 % in WT mice after 4 days of the low-K⁺ diet ($p < 0.01$) and is not modified in HKA2-null mice (Fig. 3C). It has to be noted that a cleaved band at 70 kDa should be observed when ENaC is stimulated. In our Western Blot, this band is barely visible either in normal or low-K⁺ conditions indicating that ENaC is not stimulated in any conditions. The functional activity of ENaC was assessed by testing the natriuretic effect of an acute injection (ip) of amiloride. As shown in Fig. 3D, the natriuretic effect of amiloride was decreased by 30 % in WT mice under the low-K⁺ diet compared to normal conditions ($p = 0.03$). In contrast, the natriuretic response to amiloride in HKA2-null mice was not affected by the change in dietary conditions.

Vasopressin response is blunted in HKA2-null mice at the early stage of K⁺ restriction

The loss of extracellular volume may also be explained by alteration of the process controlled by vasopressin (AVP).
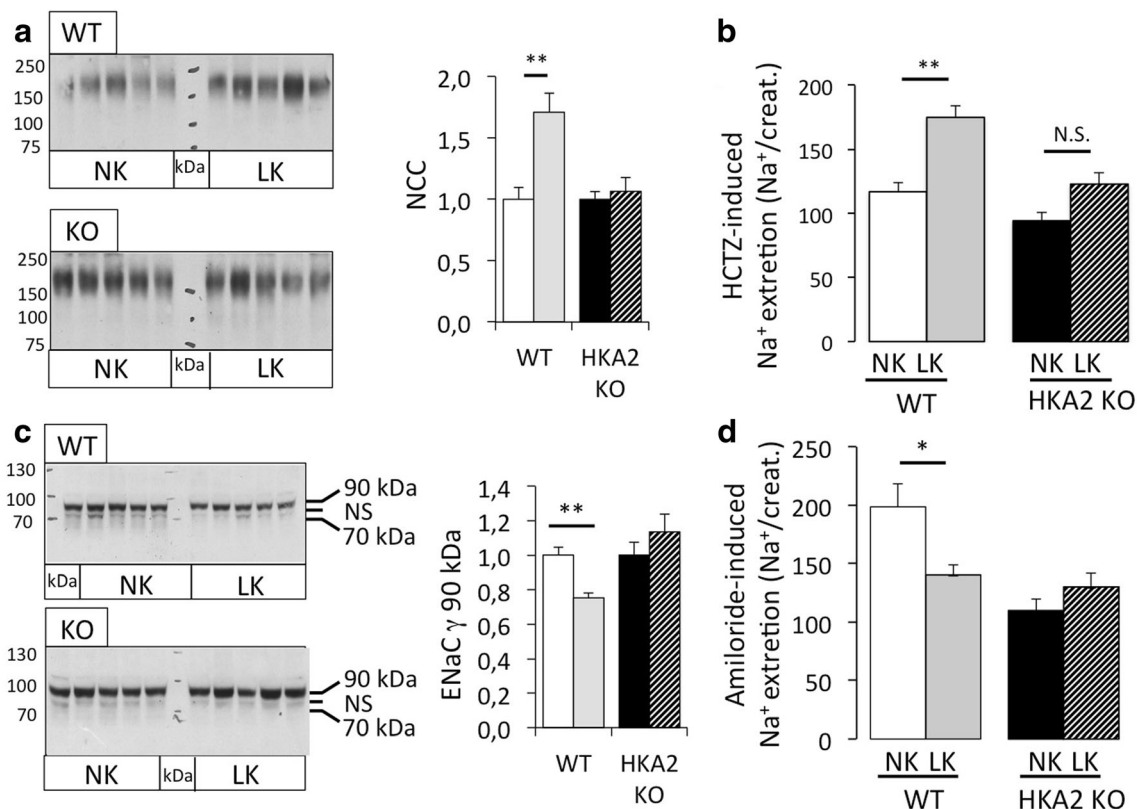

Fig. 3 *Regulation of NCC and ENaC in WT and HKA2-null mice under low-K⁺ diet.* (A) Protein expression and quantification of NCC on renal membrane preparations from WT and HKA2-null mice (HKA2 KO) under normal (NK) or low-K⁺ diets for 4 days (LK). (B) Natriuretic effects of acute (6 h) thiazide treatment performed in WT ($n = 12$) and HKA2-null mice ($n = 12$) before (NK, white bar for WT and black bar for HKA2 KO) and after 4 days of low-K⁺ diets (LK, gray bar for WT and hatched bar for HKA2 KO). This protocol has been shown [20] to not have any natriuretic effect on NCC-null mice indicating that with this dose and in this time window the only transporter affected is NCC. (C) Protein expression and quantification of ENaC γ subunit on renal membrane preparations from WT and HKA2-null mice (HKA2 KO) under normal (NK) or low-K⁺ diets for 4 days (LK). (D) Natriuretic effects of acute (4 h) amiloride treatment performed in WT (n = 12) and HKA2-null mice (n = 12) before (NK, white bar for WT and black bar for HKA2 KO) and after 4 days of low-K⁺ diets (LK, gray bar for WT and hatched bar for HKA2 KO). Results are shown as the mean ± s.e.m. Non-paired Student t-test (** $p < 0.01$; * $p < 0.05$)

We tested this hypothesis by measuring the expression of aquaporin-2 (AQP2) water channels under our different conditions. As shown in Fig. 4A, AQP2 protein (the core-glycosylated form; cg, the fully-glycosylated form; fg or both; total) level is decreased by 30 % ($p < 0.05$) in WT mice after 4 days of K^+ restriction. In HKA2-null mice (Fig. 4A), K^+ restriction induced a more severe decreased (by 55–60 %) of the expression of AQP2 (cg, fg and total protein, $p < 0.01$). Noteworthy, under normal condition, although total AQP2 level is similar between WT and HKA2-null mice, the repartition between cg and fg is different. Thus, HKA2-null mice display a higher level of cg AQP2 and a lower level of fg AQP2, possibly indicating that HKA2-null mice have lower level of AQP2 at the cell surface. To better investigate the localization of AQP2, we performed immunolabeling of AQP2 on kidney slices of WT and HKA2-null mice under normal or low K^+ diet. As shown in Fig. 4B, AQP2 displayed a sharp labeling at the apical sides of collecting duct cells in WT mice fed a normal chow. Potassium restriction slightly modified this localization. In HKA2-null mice under normal diet, the labeling was more diffuse (less sharp) but remained localized to the apical sides indicating that AQP2 may be stored in sub-membrane vesicles. Under low-K^+ diet (LK),

the localization of AQP2 was less pronounced at the apical side and exhibited a strong intracellular labeling in many cells of the HKA2-null mice CD.

To investigate further this hormonal system, we measured the acute concentrating effect of dDAVP (V2 receptor agonist) in WT and HKA2-null mice under normal and low K^+ diets. As shown in Fig. 4C, in WT animals, 4 days of the low K^+ diet did not modify urine osmolality compared to the normal diet (1412 ± 110 and 1850 ± 267 mOsmol/kg, respectively). In both dietary conditions, injection of dDAVP induced a 2-fold increase in urine osmolality (Fig. 4C and D), which reached 3151 ± 199 and 3166 ± 118 mOsmol under the normal and low K^+ diets, respectively. Therefore, in WT mice under K^+ restriction, the concentrating effect of dDAVP remains as efficient as in normal conditions. In HKA2-null mice, urine osmolality was similar under the normal and low K^+ diets (1994 ± 228 and 1895 ± 204 mOsmol/kg, respectively). As in WT mice, there was a 2-fold increase in urine osmolality after dDAVP injection under normal conditions (3600 ± 169 mOsmol/kg, Fig. 4C and D). However, under the low-K^+ diet, the dDAVP-induced increase in urine osmolality (2918 ± 127 mOsmol/kg) was significantly lower than in WT mice (1.5-fold vs 2.3-fold, $p = 0.018$ Fig. 4D). This result
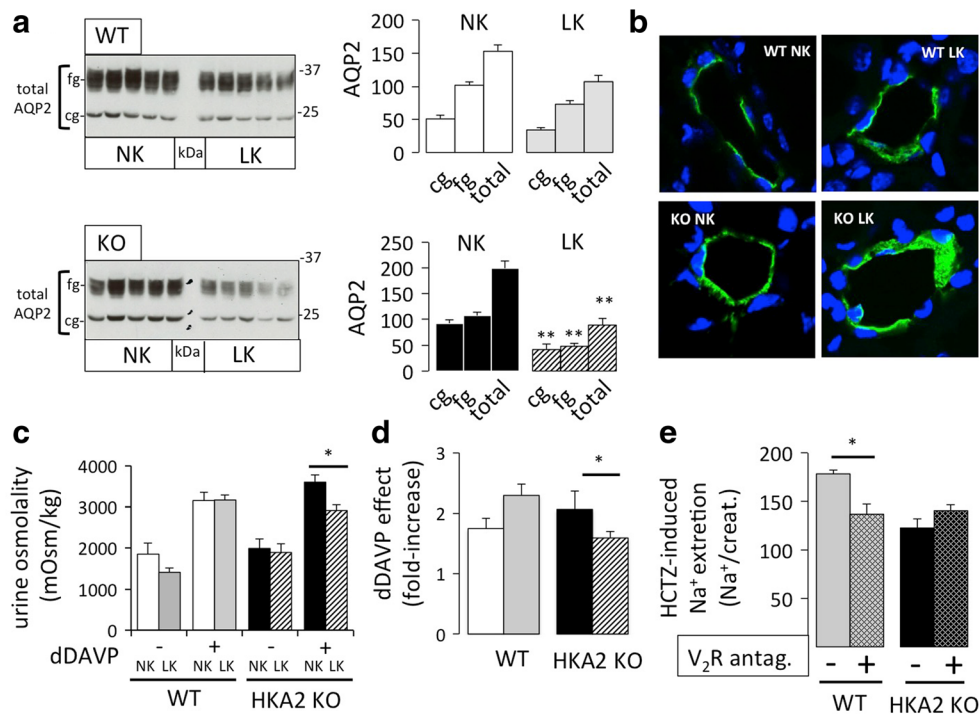

Fig. 4 *Modification of the renal water transport system by K^+ restriction.* (A) Protein expression of AQP2 in whole kidney of WT (upper panel) and HKA2-null mice (lower panel) and their quantification under normal (NK, white bars for WT and black bars for HKA2-null mice) or low-K^+ diets for 4 days (LK, gray bars for WT and hatched bars for HKA2-null mice). Results are shown as the mean ± s.e.m. ($n = 5$). Non-paired Student t-test (** p < 0.01; * p < 0.05). (B) Cell localization of AQP2 in collecting tubules of WT and HKA2-null mice under normal or low-K^+ diet. (C) Urine osmolality was measured before and 5 h after an acute injection of dDAVP to WT mice under normal (white bars) or low-K^+ diets (gray bars) or to HKA2-null mice under normal (black bars) or low-K^+ diets (hatched bars). (D) Urine osmolality measured after dDAVP injection normalized by those obtained with the same animal before the treatment. (E) Natriuretic effects of acute (6 h) thiazide treatment after 4 days of low-K^+ diets performed in WT ($n = 6$, gray bars) and HKA2-null mice (n = 6, black bars) treated (hatched bars) or not (plain bars) by a specific V2 receptor antagonist (SR121463). Results are shown as the mean ± s.e.m. Non-paired Student t-test (* $p < 0.05$)

indicates that the response to vasopressin is partially blunted in HKA2-null mice under K+ restriction.

To investigate the putative involvement of vasopressin and its V2 receptors in the stimulation of NCC during a short period of low-K+ diet, we treated our mice with a specific V2 antagonist, SR 121463 A. Under NK diet (not shown), this antagonist did not modify the response to thiazide in WT or HKA2-null mice compare to non-treated animals. However, as shown in Fig. 4E, after 4 days of LK diet, the inhibition of V2 receptors reduced the thiazide-sensitive natriuresis of WT mice but did not affect that of HKA2-null mice. This result indicates that a vasopressin-dependent process targeting the distal convoluted tubules is involved in the natriuretic response to K+-restriction but absent in HKA2-null mice.

HKA2-null mice produce PGE2 in response to K+ restriction

A possible explanation for both the decreased effect of vasopressin and expression of AQP2 in HKA2-null mice under low-K+ diet and their loss of salt may be a higher production of PGE2, a known natriuretic hormone that antagonizes the action of vasopressin [4, 16]. As shown in Fig. 5A, the mRNA of Cox-2, the enzyme that participates at the generation of PGE2 is increased by almost 50 % in kidney of HKA2-null mice. Moreover, the urinary excretion of PGE2 is 2-fold increased after 4 days of K+ restriction in HKA2-null mice but not in WT mice.

HKA2-null mice are protected against salt-sensitive hypertension induced by K+ restriction

The results above demonstrate that WT mice, in the early stage of K+ restriction, adapt their renal Na+ transport system by stimulating NCC expression and activity. This likely allows the organism to reduce K+ secretion in the more distal parts of the nephron by replacing an electrogenic transport system (ENaC) by an electroneutral one (NCC) as previously proposed [13]. Since activation of NCC may robustly enhance Na+ reabsorption, we then investigated how Na+ excretion and the blood pressure of WT and HKA2-null mice are impacted by an increase in Na+ intake after a "preconditioning" period of K+ restriction. Table 2 summarizes some physiological parameters showing that addition of NaCl in the drinking bottle did not modify the differences observed between WT and HKA2-null mice induced by K+ restriction such as lower plasma [K+] and weight in knock-out animals compared to WT. However, both strains exhibited similar urine volume and urine K+ excretion. As shown in Fig. 6A, WT and HKA2-null mice exhibit a similar Na+ intake after two days of low-K+ preconditioning and Na+ loading. Renal Na+ excretion is increased following Na+ loading in both genotype but it tends to increase even more in HKA2-null mice (Fig. 6B), which may suggest a more efficient Na+ excretion. To confirm this hypothesis, we normalized the excretion by calculating the mean ratio of the renal excreted Na+/Na+ intake (Fig. 6C) and we observed that WT mice exhibit a lower efficiency to eliminate Na+ than HKA2-null mice (70 ± 4 % vs 83 ± 4 %, $p = 0.047$ at day 4) indicating that HKA2-null mice are more prone to excrete Na+ than WT mice when preconditioned by a period low-K+ diet. We then measured the consequence of this difference in ability to excrete Na+ on the blood pressure level. After two days of K+ restriction, addition of NaCl in the drinking water (K+ restriction being maintained), significantly raised (one-way ANOVA, $p = 0.006$) BP, by roughly 15 mmHg (Fig. 6D, black circles, 141 ± 4 and 137 ± 3 mmHg, respectively) compared to day 1 and 2 (no salt supplementation, 126 ± 4 mmHg and 128 ± 3 mmHg). In other words, at the early stage of K+
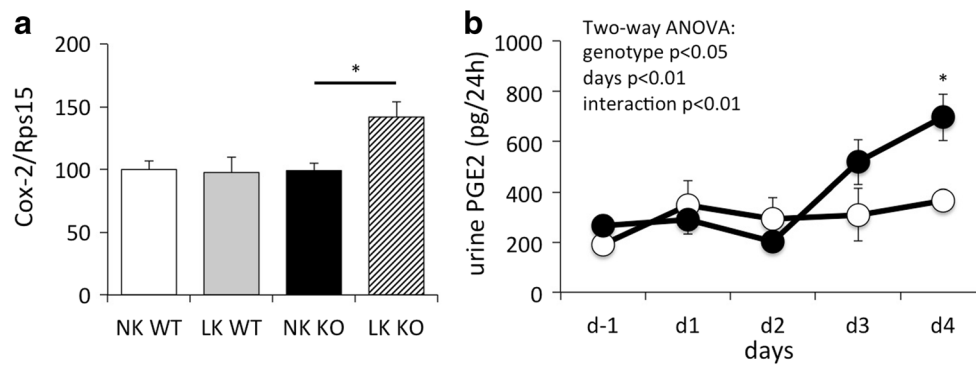

Fig. 5 *Urine PGE2 content in WT and HKA2-null mice during K+ restriction.* (A) mRNA expression of the cyclo-oxygenase isoform 2 (Cox-2) (as a percentage of the mean value obtained either for the WT mice under NK or the HKA2-null mice under NK) normalized to that of rps15, in total kidney of WT mice under a normal diet (NK, white bar, n = 9) or K+ restriction (LK, gray bar, n = 12) and of HKA2-null mice under a normal diet (NK, black bars, n = 8) or a low-K+ diet for 4 days (LK, hatched bars, n = 9). (B) Total urine PGE2 excretion in WT (white dots) and HKA2-null mice (black dots) before (d-1) and after dietary K+ restriction (d1 to d4). Results are shown as mean ± s.e.m. (n = 6) Statistical analysis was performed by a two-way ANOVA test to measure the potential effects of genotype, time of diet and their interaction followed by Bonferroni posttests to compare for each time point the WT and HKA2-null mice groups (* $p < 0.05$)

Table 2 Metabolic parameters of WT and HKA2-null mice under normal diet (NK ; n = 15 and 16, respectively) and after 2 days under LK diet and NaCl loading (2 % in drinking water) following a period of 2 days under low K^+ diet only (n = 6). Results are shown as mean ± s.e.m. HKA2-null mice vs WT, non-paired Student t-test (* $p < 0.05$)

	NK		LK-HNa (day 4)	
	WT	HKA2-KO	WT	HKA2-KO
Weight (g)	28.0 ± 0.8	27.0 ± 0.7	25.7 ± 0.7	23.4 ± 0.8*
Food intake (g)	3.1 ± 0.2	3.4 ± 0.3	2.5 ± 0.2	2.0 ± 0.3
Plasma K^+ value (mM)	4.0 ± 0.10	4.1 ± 0.2	3.4 ± 0.1	3.0 ± 0.1*
Urine volume (ml)	0.8 ± 0.1	1.1 ± 0.2	12.6 ± 2.4	13.0 ± 2.9
Urine K^+ μmol/24 h	515 ± 32	503 ± 27	12 ± 1	10 ± 4

restriction, when plasma $[K^+]$ levels were not yet affected, WT mice developed a salt-sensitive hypertension. Interestingly, the same protocol conducted in HKA2-null mice did not yield the same results. As shown previously, we observed a decrease in the BP during the first days of K^+-restriction (Fig. 6D, day 1 and 2). This decrease was not significantly altered by addition of NaCl in the drinking water (Fig. 6D, one-way ANOVA, p = 0,07) at day 3 (113 ± 3 mmHg) and day 4 (112 ± 3 mmHg) of the K^+ restriction period, compared to day 1 and 2 (no salt supplementation, 110 ± 3 mmHg and 108 ± 2 mmHg) or compared to the normal dietary situation (day-1, 117 ± 2 mmHg).

Finally, comparison of WT (Fig. 6D, circles) and HKA2-null mice (Fig. 6D, squares) clearly demonstrates that the absence of HKA2 protects against the development of this salt-sensitive hypertension. Indeed, the difference in BP between both strains is not significant under normal conditions (day-1), increases as previously shown (Fig. 1B) during the first days of K^+ depletion, and finally reaches almost 30 mmHg when salt supplementation is provided during K^+ restriction (day 3 and 4, black circles and squares). Statistical analysis by a two-way ANOVA test showed that the BP depends on genotypes ($p < 0.0001$), the type of diets ($p < 0.01$), and the interaction of both factors ($p = 0.03$).
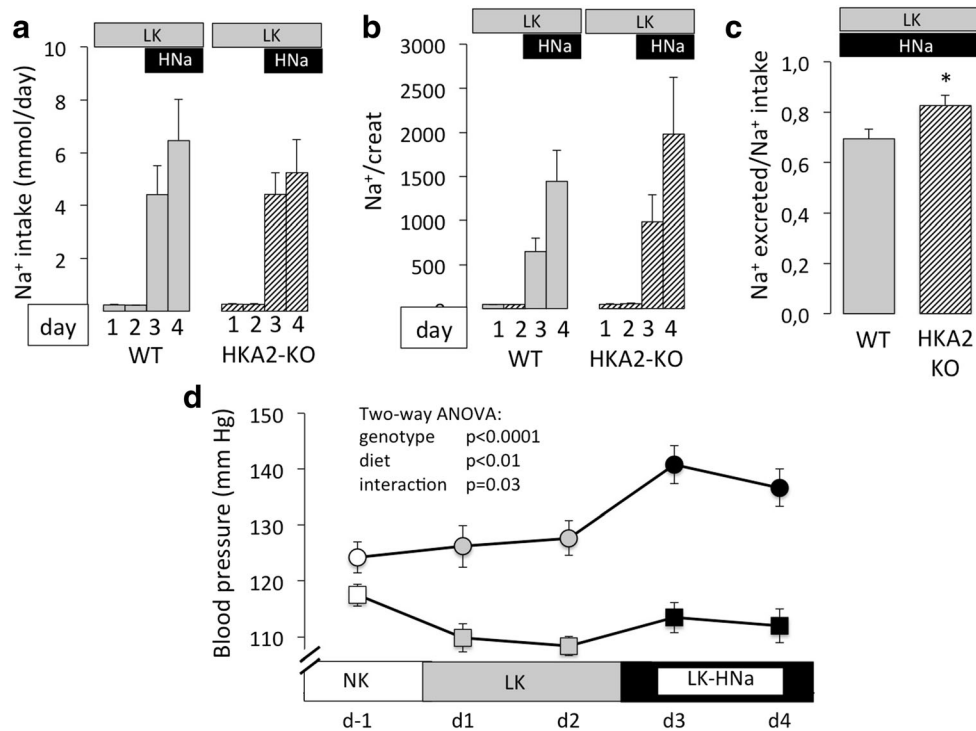

Fig. 6 *Effect of increased Na^+ intake during K^+ restriction on blood pressure level of WT and HKA2-null mice.* (A) Na^+ intake of WT and HKA2 KO mice under low-K^+ diet and challenged with a load of NaCl (n = 6). (B) Urine Na^+/creatinine excretion of WT and HKA2 KO mice under low-K^+ diet and challenged with a load of NaCl (n = 6). (C) Calculation of the Na^+ excretion efficiency (μmol excreted/μmol ingested) the fourth day of the experiment. (D) Systolic BP was measured in WT (circles) and HKA2-null (squares) mice submitted to a normal diet (white symbols), a low-K^+ diet (gray symbols) and a low-K^+/high Na^+ diet (black symbols). Results are shown as the mean ± s.e.m. (n = 12–15). Statistical analysis was performed by a one-way ANOVA test for a given genotype and by a two-way ANOVA test to measure the potential effects of genotype and diet

Discussion

In animal models, the induction of hypotension by a complete K^+ restriction has been described in many studies using different animal species [18, 21, 28], however, in humans for reasons discussed below reduction of K^+ intake is generally related to hypertension. In the present study, we tried to understand the mechanisms linking K^+ balance and the control of the BP. Interestingly, we show in this study that the absence of the HKA2 affects the regulation of volemia and BP during K^+ restriction. This could be, in part, linked to unspecific effects related to a lower body weight or to general physiological alterations induced by the depletion of K^+. However, we rather propose that the reduction of blood volume and, consequently, of blood pressure is a physiological response that helps maintaining the plasma K^+ level in the HKA2-null mice as we will discuss below.

Short-term adaptation to K^+ restriction requires modification of the renal Na^+ transport system in wild-type animals

It is well established that a low-K^+ diet induces a fall in aldosterone production that results in a decrease in ENaC expression [13] and in vivo activity (this study). In addition, the decreased Na^+ transport in the thick ascending limb of K^+-depleted animal reported years ago [17] was associated by Amlal et al. [1] to the reduced mRNA expression as well as the low activity of NKCC2. ENaC and NKCC2 inhibition should lead to a major loss of Na^+ and fluid in the urine. However, K^+-depleted WT animals only slightly increase their urinary volume and Na^+ excretion and therefore seem to compensate these diuretic and natriuretic effects. Here, we showed that this compensation involves an increase in the expression and activity of NCC and requires the preservation of an efficient vasopressin system. The stimulation of total NCC expression during a low-K^+ diet has been reported by Palmer's group in rats [13] but not by Nguyen et al. [28]. Recently, Castenada-Bueno et al. [3] as well as Wade et al. [40] observed, in WT mice under the same dietary conditions as ours (4 days of a low-K^+ diet), a similar increase in total NCC protein expression. We did not determine the level of pNCC but the natriuretic effect of thiazide (with a protocol targeting specifically NCC and not the pendrin/NDCBE electroneutral transporters [20]) was clearly more important in WT mice under a low-K^+ diet, which indicates indeed increased activity of this transporter. It is therefore likely that the stimulation of NCC activity during a low-K^+ diet helps to compensate for the decrease in NKCC2 and ENaC expression/activity. Interestingly, Morris et al. [26] reported that the absence of NCC provokes a higher loss of fluid during K^+ restriction, underlying the importance of this transporter to maintain extracellular volume levels in this given condition. More

recently, Tecker et al. [37]. confirmed the role of NCC activation during K^+ restriction and demonstrated that NCC-null mice do not develop hypertension in this context.

Is vasopressin the key hormone to preserve extracellular volume during a low-K^+ diet?

Interestingly, in their study, Castaneda-Bueno et al. showed that NCC activation under K^+ restriction (WNK4-independent) is different than that mediated by angiotensin II infusion (WNK4-dependent). Since OSR1 (which may compensate for the absence of SPAK under a low-K^+ diet) is phosphorylated after a vasopressin infusion [31, 35], this hormone may be the trigger for promoting water preservation and stimulation of NCC. To support this hypothesis, we showed, in the present study, that in the context of K^+ restriction, the inhibition of the vasopressin pathway depending on V2 receptors impedes the stimulation of NCC. Conversely, in HKA2-null mice under K^+ restriction, we showed that vasopressin was less efficient in elevating urine osmolality and that AQP2 protein level was more severely decreased than in WT and that V2 receptor antagonism has no effect on NCC activity. All these results indicate that the vasopressin system is blunted in these animals after 4 days of a low-K^+ diet. Interestingly, in this context, the plasma $[K^+]$ of HKA2-null mice is 20 % lower than in wild-type animals (Table 1), leading to a clear hypokalemia and modification of this parameter may interfer with vasopressin production. Some reports suggest that an increase in K^+ intake [10] or in plasma $[K^+]$ [38] could stimulate the production or release of vasopressin. Conversely, K^+-depleted rats displaying hypokalemia exhibit a blunted response to vasopressin injections [27]. These results indicate a relationship between the level of plasma $[K^+]$ and vasopressin action. Therefore, the marked hypokalemia occurring in HKA2-null mice after a short period of K^+ restriction may explain the reduced effects of vasopressin. A mechanistic reason for the blunted response to vasopressin remains to be investigated in more detail, but we propose that the increased production of PGE2, a factor that antagonizes vasopressin effects in different segments [4, 16], is a possible explanation.

Low K^+ diet and salt-sensitive hypertension

A recent report suggests that a decrease of plasma $[K^+]$ could trigger the decrease of intracellular $[Cl^-]$ and in fine result in activation of NCC through WNK4 [37]. Our physiological observations do not fully support this idea. Indeed, a decrease in K^+ intake that does not affect plasma $[K^+]$ (our WT mice after 4 days of LK diet as well as in [3]) already activates NCC and induces a salt-sensitive hypertension. It seems therefore not necessary to reach a state of hypokalemia to activate thiazide-sensitive Na^+ transport in the DCT. We, also, described a situation where the development of hypokalemia

(as in the HKA2-null model) does not activate NCC. The mechanisms that regulate NCC under K$^+$-deficiency are therefore more complicated.

Numerous studies indicate that the daily consumption of K$^+$ in westernized populations is roughly 30 % lower than that which is recommended (4.7 g or 120 mEq) by the Food and Nutrition Board of the Institute of Medicine of the National Academies (http://fnic.nal.usda.gov/dietary-guidance/dri-nutrient-reports/water-potassium-sodium-chloride-and-sulfate). It is however likely that these whole "healthy" populations are not prone to hypokalemia. A recently published association study [23] showed that a slight decrease in K$^+$ excretion (reflecting a lower K$^+$ intake) raises blood pressure and that this effect is dependent on Na$^+$ excretion (also reflecting Na$^+$ intake). In this cohort, the variations in urine K$^+$ excretion (or K$^+$ intake) was moderated (mean 2.12 ± 0,60 g/day) and was not likely to be associated with a significant modifications of plasma [K$^+$] although this parameter was not measured. The groups of people with a lower K$^+$ excretion (i.e., a lower K$^+$ intake) were in a situation likely to correspond to our WT mice after a short period of almost complete K$^+$ restriction, i.e. in a situation where their plasma [K$^+$] is in the normal range and their NCC is activated. This activation could explain why, in this group, as in our WT K$^+$-depleted mice, an increase in Na$^+$ intake raises blood pressure. This comparison is however limited since we abruptly and strongly modified the diet composition of our mice and only controlled prospective studies in human with normalized low-K$^+$ and high-Na$^+$ diets intake would help confirming this hypothesis.

Importance of the HKA2 in the global response to K$^+$ restriction

Physiological adaptation to K$^+$ restriction requires a functional HKA2 in both the colon and kidney [12, 22]. In the present study, we showed that stimulation of HKA2 by delaying the decrease of plasma [K$^+$] also helps at maintaining the volume of the extracellular compartment since its absence promote both hypokalemia and hypotension.

In this particular situation, since the strongest threat is the development of a non-manageable hypokalemia, in order to control the decline in plasma [K$^+$], the organism reduces its extracellular volume to the detriment of blood pressure. This process may be considered as a positive adaptation, as it allows the organism to concentrate K$^+$ in the extracellular compartment and therefore to maintain plasma [K$^+$] in a bearable range.

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