Published in final edited form as:
Hypertension. 2012 February ; 59(2): 339–347. doi:10.1161/HYPERTENSIONAHA.111.178475.

Cyp2c44-epoxygenase is essential for preventing the renal sodium absorption during increasing dietary potassium (K)-intake

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Abstract

The aim of this study is to test whether the Cyp2c44 epoxygenase dependent metabolism of arachidonic acid (AA) prevents the hypertensive effect of a high K intake (HK) by inhibiting the ENaC activity. A HK intake elevated Cyp2c44 mRNA expression and 11,12-epoxyeicosatrienoic acid (11,12-EET) levels in the cortical collecting duct (CCD) in *Cyp2c44(+/+) mice(wt)*. However, a HK intake failed to increase 11,12-EET formation in the CCDs of *Cyp2c44(-/-)* mice. Moreover, increasing K intake enhanced AA-induced inhibition of ENaC in the wt but not in *Cyp2c44(-/-)* mice. In contrast, 11,12-EET, a Cyp2c44 metabolite, inhibited ENaC in the wt and *Cyp2c44(-/-)* mice. The notion that Cyp2c44 is the epoxygenase responsible for mediating the inhibitory effects of AA on ENaC is further suggested by the observation that inhibiting Cyp-epoxygenase increased the whole-cell Na currents in principal cells of wt but not in *Cyp2c44(-/-)* mice. Feeding mice with a HK diet raised the systemic blood pressures of *Cyp2c44(-/-)* mice but was without an effect on wt mice. Moreover, application of amiloride abolished the HK-induced hypertension in *Cyp2c44(-/-)* mice. The HK-induced hypertension of *Cyp2c44(-/-)* mice was accompanied by decreasing 24-hr urinary Na excretion and increasing the plasma Na concentration, the effects were absent in wt mice. In contrast, disruption of the *Cyp2c44* gene did not alter K excretion. We conclude that Cyp2c44 epoxygenase mediates the inhibitory effect of AA on ENaC and that Cyp2c44 functions as a HK-inducible anti-hypertensive enzyme responsible for inhibiting ENaC activity and Na absorption in the aldosterone-sensitive distal nephron (ASDN).

Keywords

11,12-EET; Arachidonic acid; ENaC; hypertension; kidney

Introduction

Increasing the dietary K intake has been reported to prevent the high salt intake-induced hypertension in both humans and rats [1-3]. The guideline published in a DASH diet (Dietary Approaches to Stop Hypertension) recommends all healthy adults to double their daily K intake to prevent salt-sensitive hypertension. However, the underlying mechanisms by which a HK intake prevents the salt-sensitive hypertension are not completely understood.

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Disclosures None

Factors such as kallikrein [4] and prostaglandins [5] have been suggested to play a role in decreasing blood pressure (BP) and increasing renal Na excretion. Our previous study demonstrated that a HK intake stimulated the kidney expression of the rat Cyp2c23-epoxygenase, a homologue of the murine Cyp2c44 epoxygenase [6]. A large body of evidence indicates that CYP2C-epoxygenase-dependent AA metabolism plays an important role in preventing the salt-sensitive hypertension [7-12]. Inhibition of renal CYP2C-epoxygenases caused hypertension in rats on high Na diets [7, 8]. However, the animals became normotensive after the removal of the epoxygenase inhibitor, even when the animals were kept on a high Na diet. The role of the epoxygenase in preventing salt-sensitive hypertension was best demonstrated in the *Cyp4a10(-/-)* mouse model [12]. Although Cyp4a10 does not metabolize AA to EETs [6], disruption of the *Cyp4a10* gene reduced the expression of epoxygenase such as Cyp2c44 and resulted in salt- sensitive hypertension (11). However, the identity of the anti-hypertensive epoxygenase isoform(s) is not unambiguously determined. Because Cyp2c44 homologues are epoxygenase isoforms responsible for 11,12-EET biosynthesis in the kidney [7, 9, 13, 14] and their expression is upregulated by a HK intake [15], we test whether Cyp2c44-dependent metabolism of AA plays a role in mediating the antihypertensive effects of a HK-intake.

Results

We previously demonstrated that increasing dietary K content from 1% to 10% stimulated the renal expression of Cyp2c23 (the homologue of mouse Cyp2c44) and 11,12-EET biosynthesis in the isolated CCD of rat kidney [19]. We now examined whether an increase in the dietary K within physiological relevant ranges was sufficient to stimulate Cyp2c44 expression in kidney. Fig. 1A is a Western blot analysis showing that increasing dietary K intake from 1% to 2.5% for 7 days stimulated the expression of Cyp2c44 by 50±10% (n=4) in the kidney tissue of wt mice (The tissue is from cortex and outer medulla region). Previous studies demonstrated that mouse Cyp2c44 and rat Cyp2c23 were highly expressed in the CCD [12, 18]. Thus, we examined the effect of a HK intake on Cyp2c44 mRNA levels by real time PCR in the isolated mouse CCD using Cyp2c44 specific PCR primers. Increasing dietary K content from 1% to 2.5% stimulated the levels of Cyp2c44 mRNA by 4 fold in the mouse CCD (N=5) (Fig.1B).

The CYP epoxygenases metabolize AA to 5,6, 8,9, 11,12 and 14, 15- EET [6] and the 11,12-EET accounts for over 60 % of the total microsomal epoxidation of AA in the kidney [7]. To examine whether the disruption of the Cyp2c44 gene altered the effects of a HK intake on 11,12-EET generation in the CCD, we measured the concentrations of 11,12-EET and its product, the 11,12-dihydroxyeicosatrienoic acid (11,12-DHET) in the CCDs isolated from wt and *Cyp2c44*-/- mice, respectively. As shown in Fig 1C, a HK intake increased the levels of 11,12-EET and 11,12-DHET in the CCDs of the wt mice from 260± 30 to 500±80 pg/mg protein (N=4). In contrast, under similar conditions, increasing K content from 1% to 2.5% failed to raise the levels of 11.12-EET and 11,12-DHET in the CCDs of *Cyp2c44(-/-)* mice (control, 120±18 pg/mg protein; HK, 90±12 pg/mg protein; N=4). These results identify Cyp2c44 as the epoxygenase responsible for the increases in 11,12-EET biosynthesis of the CCD in response to a HK intake.

Since non-Cyp2c44 epoxygenases are also expressed in the CCD [20], we next examined whether the inhibitory effects of AA on ENaC were Cyp2c44-dependent by analyzing ENaC responses to AA in the CCD dissected from wt or *Cyp2c44(-/-)* mice fed a control K or HK diet. Fig. 2A is a channel recording in a cell-attached patch showing that the application of AA (15 μM) did not inhibit ENaC activity in *Cyp2c44*(-/-) mice. In ten similar experiments, the channel activity, defined by NP_o, was not significantly different (1.94±0.6) in the presence of or in the absence of AA (10-15 μM) (1.8±0.6, Fig. 2B). In contrast, increasing K

content from 1% to 2.5% enhanced AA-mediated inhibition of ENaC in the wt mice. Data summarized in Fig. 2B demonstrate that application of AA (10 μM) inhibited ENaC activity from 0.87±0.04 to 0.4±0.06 (top panel) or by 52±5% (low panel) in the wt mice on a control K diet (N=8), and reduced NP_o from 1.9±0.4 to 0.23±0.06 or by 88±10% in the animals fed a HK diet (N=8). The lack of an AA effect on the ENaC activity in *Cyp2c44(-/-)* mice was due to the absence of Cyp2c44 epoxygenase activity because 100 nM 11,12-EET (Cyp2c44 AA metabolite) blocked ENaC in the CCD of *Cyp2c44(-/-)* on both control and HK diet (data not shown). These findings are consistent with the previous report that AA-mediated inhibition of ENaC was enhanced in the CCD of rats on 10% K diet [15]. Taken together, the results suggest that Cyp2c44 is the epoxygenase responsible for mediating the effect of AA on ENaC in the mouse CCD.

We next examined whether Cyp2c44-dependent AA metabolism was involved in controlling ENaC basal activity by measuring the amiloride-sensitive Na currents in principal cells with perforated whole-cell recordings in the CCD of wt and *Cyp2c44(-/-)* mice on a HK diet for 3-7 days. We suspect that inhibition of CYP-epoxygenase might increase ENaC activity if Cyp2c44 is involved in regulating channel basal activity. Fig. 3A is a whole-cell recording showing that treatment of principal cells with MS-PPOH (5 μM), an inhibitor of Cyp-epoxygenase [21], increased amiloride-sensitive Na currents in principal cell from 246±50 pA to 429±76 pA ($P<0.05$, N=5) at -100 mV in the wt mice (Fig 3A and 3B, left panel), but not in *Cyp2c44(-/-)* mice (Fig 3A, right panel). Fig. 3B shows an I/V curve generated from 5 experiments demonstrating the effect of epoxygenase inhibition on the amiloride-sensitive Na currents in principal cells of the CCD from the wt (left panel) and *Cyp2c44(-/-)* mice on a HK diet (right panel). From inspection of Fig. 3B, it is apparent that the disruption of the *Cyp2c44* gene increased amiloride-sensitive Na currents in principal cells from 246±50 pA (wt mice) to 482±90 pA ($P<0.05$) at -100 mV (*Cyp2c44*-/- mice). Furthermore, inhibition of Cyp-epoxygenase with MS-PPOH failed to increase Na currents in *Cyp2c44(-/-)* mice (489±140 pA at -100 mV). Similar results were observed in the mice fed a normal diet (1% K+0.3% Na) (please see http://hyper.ahajournals.org, Supplemental Fig. S3). Inhibition of epoxygenase increased whole-cell Na currents in wt but had no effect in *Cyp2c44(-/-)* mice. Therefore, results strongly suggest that Cyp2c44-dependent AA metabolism is responsible for suppressing ENaC activity in principal cells of the wt mice fed a HK diet.

Since ENaC activity is upregulated in the CCD from *Cyp2c44(-/-)* mice on a HK diet, it is conceivable that increasing dietary K intake may enhance renal Na absorption or decrease the renal Na excretion in the mice containing disrupted *Cyp2c44* gene. Thus, we measured the food intake and urinary excretion of Na and K over a 24 hr period with metabolic cages in the wt and *Cyp2c44(-/-)* mice fed a normal K diet for 3 days and then fed a HK diet for an additional 3 days. Results summarized in Fig. 4 show the 24-hr dietary Na intake and urinary Na excretion (UNa) in the wt and *Cyp2C44(-/-)* mice on a normal K diet (left panel) and on a HK diet (right panel). It is apparent that the ratios between UNa and dietary Na intake were not significantly different between wt and *Cyp2c44(-/-)* mice fed a normal K diet for three days (0.75 ±0.04 and 0.68±0.04, for wt and *Cyp2c44(-/-)* mice, respectively; N=8). These results suggest that disruption of the *Cyp2c44* does not significantly alter renal Na handling in animals under control conditions. However, when fed a HK diet, UNa for *Cyp2c44(-/-)* mice was significantly lower than those of wt animals. The ratio for day 3 between UNa and Na intake for 24 hr decreased from 0.68±0.04 (control diet) to 0.43±0.04 (HK diet, N=8) in *Cyp2C44-/- mice* while the ration was unchanged in the wt animals on a HK diet (0.76±0.04). Therefore, lack of a functional Cyp2c44 epoxygenase impairs the renal ability to excrete Na during increasing dietary K intake. Next, we used a similar experimental protocol to examine whether the urinary excretion of K was altered in mice carrying a disrupted *Cyp2c44* gene. Fig. 5 summarizes experiments in which 24-hr dietary K intake and 24-hr urinary K excretion (UK) were examined in wt (N=8) and in *Cyp2c44(-/-)*

mice (N=8) on a NK or on a HK diet. From inspection of Fig.5, it is apparent that disruption of *Cyp2c44* gene did not significantly affect renal ability of K secretion under both control conditions and during a HK intake. The ratios between 24-hr UK and K intake in mice on a HK diet for day 3 were 0.65±0.04 (wt) and 0.8 ±0.04 (*Cyp2c44*-/-), respectively.

The notion that disruption of the *Cyp2c44* gene impairs Na but not K excretion during increased dietary K intake is supported by measurements of plasma Na concentrations in wt and *Cyp2c44(-/-)* mice fed a normal or a HK diet for three days. As shown in Fig.6A, plasma Na concentrations were not significantly different for wt and *Cyp2c44*-/- mice fed a control K diet (wt, 151±1 mM; *Cyp2c44*-/-, 151.2±2.5 mM). However, when fed a HK diet, plasma Na levels were significantly higher in *Cyp2c44(-/-)* mice than those of wt mice (150±1 and 156.9±1 mM, for wt and *Cyp2c44(-/-)* mice, respectively; N=5). As consequence of high plasma Na concentration and a low UNa, *Cyp2c44(-/-)* mice gained weight from 29.2±0.4 g to 32.6±0.7 g ($P<0.05$, N=4) while, in contrast, the wt animals lost weight from 28.2 ±0.2 g to 26.0 ±1 g ($P<0.05$, N=4) one day after animals were fed with a HK diet (Fig.6B). The above results suggest that a HK diet causes the volume expansion in *Cyp2c44(-/-)* but not in wt animals. This notion is supported by the finding that the HK-induced increase in aldosterone level was suppressed in *Cyp2c44(-/-)* animals, presumably as the results of volume expansion, in comparison to the wt mice. Although the aldosterone levels were similar under control condition (wt, 550±50 pg/ml, *Cyp2c44(-/-)*,450 ±50 pg/ml), a HK intake for 3 days increased the plasma aldosterone in wt mice (1500± 300 pg/ml), a value was significantly higher than those in the *Cyp2c44(-/-)* animals (900 ±200 pg/ml, N=4).

To determine whether a decrease in UNa caused hypertension in *Cyp2c44(-/-)* mice, we measured on a daily basis the BP of wt and *Cyp2c44(-/-)* mice fed diets containing either normal (1% KCl) or HK (2.5% KCl) from 1 to 3 days. When fed a normal K, the systolic BPs of wt and *Cyp2c44(-/-)* were not significantly different (125 ±4 and 122 ±7 mm Hg for wt and *Cyp2c44*-/- mice, respectively) (Fig 7A). However, within the first 24 hour of exposure to a HK diet the BP of *Cyp2c44(-/-)*, but not wt mice, increased markedly (to 166 ± 7 mm Hg) (N=8) and remained high as long as the mice were fed with the HK diet (Fig 7A and also please see http://hyper.ahajournals.org, Supplemental Fig. S4). In contrast, under the same conditions, the HK diet had no significant effects on BP of wt mice, and the animals remained normotensive for the duration of the experiment (Fig 7A and supplemental Fig.4). Thus, increasing dietary K intake causes hypertension only in Cyp2c44-/- mice. Moreover, the HK-induced hypertension in CYP2c44-/- mice was the results of high ENaC activity because application of amiloride (0.5 mg/100 g body weight) abolished the effect of a HK diet on the BP in CYP2C44-/- mice (Fig.7B).

Discussion

It is well documented that metabolism of AA by CYP-epoxygenases plays a role in preventing salt-sensitive hypertension in animal models [7-12]. A high salt intake has been shown to stimulate Cyp2c23 expression in rats and a decrease in renal epoxygenase activity and urinary EETs levels is related to hypertension in Dahl rats [7, 8]. CYP-epoxygenase **-** metabolites may prevent salt-sensitive hypertension by modulating renal hemodynamics and by inhibiting renal Na transport. It has been reported that 11,12-EET regulates glomerular afferent arterioles thereby possibly increasing glomerular filtration rate and facilitating Na excretion [22-24]. In addition, inhibition of renal Na absorption by the CYP-epoxygenase-dependent AA metabolism could play a role in preventing the salt-sensitive hypertension. For instance, 5,6-EET has been shown to decrease Na absorption in the CCD by inhibiting the Na/H exchanger in the rabbit kidney [25]. We have previously demonstrated that CYP-epoxygenase dependent AA metabolites inhibited ENaC activity in the CCD [15, 18, 26] and

that 11,12-EET mediated the inhibitory effect of AA on ENaC activity [15]. The effect of 11,12-EET was specific because EETs other than 11,12-EET had either no effect or exerted a modest inhibitory effect on ENaC [18]. The role of CYP-epoxygenase in preventing salt-sensitive hypertension has been documented in *Cyp4a10(-/-)* mice which developed hypertension when fed a high salt diet and have impaired regulatory control of their Cyp2c44 epoxygenase by dietary salt [12]. Two lines of evidence strongly suggest that defective regulation of ENaC by epoxygenase-dependent AA metabolism was responsible for the salt intake-induced hypertension in CYP4A10-/- mice: 1) patch-clamp experiments demonstrated that AA failed to inhibit while 11,12-EET was able to block ENaC in the CCD of CYP4A10-/- mice, 2) inhibition of ENaC with amiloride abolished the salt-sensitive hypertension in CYP4A10-/- mice on a high salt diet.

Although the role of CYP-epoxygenase-dependent AA metabolism in regulating ENaC is well established, it is still not understood which CYP-epoxygenase is mainly responsible for regulating ENaC activity. Disruption of the *Cyp4a10* gene compromised renal EET biosynthesis [12], suggesting that epoxygenases other than Cyp2c44 could also be down-regulated in Cyp4a10-/- mice. Three lines of evidence strongly suggest that Cyp2c44 is the epoxygenase responsible for mediating the inhibitory effect of AA on ENaC activity: 1) the whole-cell Na current in principal cell of the CCD was significantly higher in *Cyp2c44(-/-)* mice than those of the wt mice; 2) inhibition of epoxygenase increased ENaC activity in the wt but not in *Cyp2c44(-/-)* mice; 3) AA failed to inhibit while 11,12-EET blocked ENaC activity in *Cyp2c44(-/-)* mice. These results strongly indicate that Cyp2c44 is the epoxygenase responsible for mediating AA-induced inhibition of ENaC.

In addition to high salt intake, our previous and present studies have also demonstrated that a HK intake stimulates the expression of Cyp2c44 in the kidney especially in the CCD [15]. Moreover, the effect of a HK intake on Cyp2c44 was specific because a HK intake failed to increase CYP2J2 expression which is expressed in the CCD [20]. The observation that a HK intake failed to increase 11,12-EET/DHET generation in the isolated CCD of *Cyp2c44(-/-)* mice strongly suggests that Cyp2c44 is the epoxygenase responsible for mediating the effect of a HK intake on 11,12-EET generation.

Increasing dietary K intake is expected to stimulate aldosterone secretion which activates Na-K-ATPase and ENaC activity in ASDN [27-29]. However, a high aldosterone induced by a high K diet did not increase renal Na absorption in ASDN and did not cause hypertension. Instead, increased dietary K intake facilitated renal Na excretion and decreased BP [1, 30, 31]. This is partially due to a decrease in Na-Cl co-transport (NCC) expression in the distal convoluted tubule (DCT) [32] thereby reducing Na absorption in the DCT. In addition, we suggest that the HK-induced antihypertensive and natriuretic effects critically depend on Cyp2c44-dependent AA metabolism in the ASDN. The notion is supported by three lines of evidence:1) increasing dietary K content from 1% to 2.5% raises the BP in *Cyp2c44(-/-)* mice but not in the wt mice; 2) the 24 hr-urinary Na excretion was lower in *Cyp2c44(-/-)* mice on a HK diet than those of wt animals on a HK diet; 3) plasma Na concentrations and body weight were significantly higher in *Cyp2c44(-/-)* mice on a HK diet than those of the wt mice. We hypothesize that A HK intake specifically stimulates Cyp2c44 expression in the ASDN including CCD (please see http://hyper.ahajournals.org, Supplemental Fig. S5). Increased Cyp2c44 expression enhances AA metabolism to generate 11,12-EET which inhibits ENaC. Therefore, we propose that although a HK intake increases plasma aldosterone levels which stimulate ENaC expression and Na-K-ATPase, the HK-intake induced increase in 11,12-EET biosynthesis suppresses ENaC activity thereby facilitating Na excretion in ASDN and preventing hypertension. However, it is possible the mechanism other than stimulation of ENaC activity may also contribute to the HK-intake-induced increase in blood pressure in Cyp2c44-/- mice. It has been reported that a HK intake

increased the plasma vasopressin concentration in male rats [33]. Further experiments are required to explore whether a high vasopressin level is also responsible for raising the blood pressure in animals with down-regulated epoxygenase activity.

Another finding of the present study is that *Cyp2c44* disruption did not affect the renal ability to excrete K since the ratios between 24-hr urinary K secretion and dietary K intake in *Cyp2c44(-/-)* mice were similar to those wt mice under control conditions and during a HK adaptation. We have previously demonstrated that 11,12-EET activates the Ca^{2+}-dependent big-conductance K (BK) channel in the CCD thereby mediating BK channel-dependent and flow-stimulated K secretion [19]. Two factors may play a role in stimulating K secretion in *Cyp2c44(-/-)*mice. First, a HK intake is known to stimulate ROMK channel expression [32, 34-36] thereby compensating the function of BK channels. Relevant to this proposal was the report that renal K secretion was not compromised in BK channel knockout mice [37]. Second, increased ENaC activity in *Cyp2c44(-/-)* mice augments the driving force for K secretion through ROMK channels. Thus, although a decrease in 11,12-EET generation is expected to diminish the BK channel activity, K secretion in *Cyp2c44(-/-)* mice is normal through increasing ROMK channel activity and enhanced driving force for K secretion.

In summary, the present study demonstrated that 11,12-EET generated by Cyp2c44 is involved in suppressing renal Na transport in the ASDN during increasing dietary K intake. We conclude that Cyp2c44-dependent AA metabolism plays a key role in mediating HK-induced antihypertensive effect and also in promoting renal Na excretion.

Perspectives

The present study suggests a potential caveat to increase K intake for people who have defective Cyp-epoxygenase function due to genetic or pharmacological reasons because a HK intake could possibly result in dietary K sensitive hypertension. In this regard, it has been reported that polymorphisms of human Cyp2c8 and Cyp2c9 gene (functional homologues of Cyp2c44) result in a low epoxygenase activity [38]. Thus, the present study has the physiological and clinical importance because it will provide guidance for K supplement.

Methods

Animals

Isogenic Cyp2c44(-/-) and *Cyp2c44(+/+)* mice were obtained from Dr. J. Capdevila, Vanderbilt University. Global *Cyp2c44* mouse knockouts (50:50 *C57Bl/6* albino/*129 SvE*) from Lexicon Genetics Inc (Texas) were derived from an Ommibank ES (*129SeV*) cell line (clone OST85045) in which, insertion of a viral gene trap at exon 4 of *Cyp2c44* (66 bp from the upstream intro/exon boundary) generates truncated, non-coding, Cyp2c44 transcripts. These animals were crossed with *Cyp2c44(+/+)(129SeV)*(Taconic Farms, NY) to generate homozygous *Cyp2c44(+/+)* and *Cyp2c44(-/-)* mice in isogenic *129/SeV* backgrounds from the progeny of an F15 cross of heterozygous *Cyp2c44(+/-)* mice). Tail DNAs were genotype by PCR with primers flanking the trap insertion site (forward, 5′-caccttcatcctggcctgtg-3′; reversed, 5′-ttacgactgagccacattcc-3′), and a primer specific for the 3′-end of the viral trap (forward, 5′-ggcgttacttaagctagcttgc-3′).

Dissecting CCD and patch-clamp

Several thin slices of the kidney (<1 mm) were cut and placed on an ice-cold Ringer solution. After the isolation of the CCDs, they were placed on a 5 × 5mm cover-glass coated with polylysine and then transfered to a chamber (1000 μl) mounted on an inverted Nikon microscope. The CCD was cut open with a sharpened micropipette to expose the apical

membrane and was superfused with HEPES buffered NaCl solution. A borosilicate glass (1.7-mm OD) was used to make the patch-clamp pipettes that were pulled with a Narishege electrode puller. An Axon200B patch-clamp amplifier was used to record the channel current. The currents were low-pass filtered at 50 Hz and digitized by an Axon interface (Digidata 1322). Data were analyzed using the pClamp software system 9 (Axon). Channel activity, defined as NP_o, was calculated from data samples of 60 seconds duration in the steady state as follows:

$$NP_o = \sum (t_1 + 2t_2 + \ldots\ldots it_i)$$

where t_i is the fractional open time spent at each of the observed current levels. If we compare the channel activity between the mice with different treatments, we normalized data by taking the initial control values as 100%. The pipette solution for studying Na channels contained (in mM) 140 NaCl, 1.8 $MgCl_2$, 1.8 $CaCl_2$ and 5 HEPES (PH=7.4). The bath solution for single channel patch-clamp experiments contained (in mM) 135 NaCl, 5 KCl, 1.8 $CaCl_2$, 1.8 $MgCl_2$, 2 glucose and 10 HEPES (pH=7.4).

For the whole-cell clamp measurements, The CCDs were superfused with solutions containing (in mM) 135 Na methanesulfonate, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 2 glucose, 5 mM $BaCl_2$, and 10 HEPES adjusted to pH 7.4 with NaOH. The tip of the pipette was filled with pipette solution followed by backfilling with amphotericin B (2 μg/0.1 ml) containing the pipette solution which was composed of (in mM) 7 KCl, 123 aspartic acid, 20 CsOH, 20 TEAOH, 5 EGTA, 10 HEPES, 3 MgATP, and 0.3 NaGDP-βS with the pH adjusted to 7.4 with KOH. The whole-cell Na current was determined by adding 10 μM amiloride in the bath solution. Data were analyzed using the pClamp software system 9.0 (Axon).

Tissue preparation and Western blot—The renal cortex and outer medulla were separated under a dissecting microscope and suspended in RIPA solution (1:8 ratio, w/v) containing 50 mM Tris-HCl (pH=7.4), 10 mM NaCl, 1% NP-40, 1% Triton X-100, 0.1% SDS, 1 mM sodium molybdate, 1 mM para-nitrophenyl-phosphate and 1mM EDTA. For every 125 mg tissue sample, we added 25 μl cocktail of protease and phosphatase inhibitors containing aprotinin (1 μg/ml), leupeptin (1 μg/ml), pepstatin A (1μg/ml), sodium vanadate (Na_3VO_4) (1.5 mM) and sodium fluoride (1 mM). The samples were left on ice for 15 min and homogenized with a mortar and pestle. The protein concentrations were measured twice using the Pierce BSA protein assay. The homogenized tissue samples were incubated in the presence of DNAse (5 ng/ml) and rabbit IgG serum at 4°C for 60 min. The mixture was then centrifuged at 3000 rpm for 10 min at 4°C and the resultant supernatant collected. The proteins were separated by electrophoresis on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membrane. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS), rinsed and washed with 0.05% Tween20-TBS buffer. An Odyssey infrared imaging system (LI-COR, Lincoln, Nebraska) was used to scan the membrane at a wave-length of 680 or 800 nM.

Measurement of EET

The isolated CCDs were placed in a tube containing ice-cold Na Ringer (0.5 ml). Eicosanoids in the tubule and media were acidified to pH 4.0 with 9% formic acid. We added 2 ng D_8 11,12-EET in the tube as internal standard, the samples were extracted twice with 2X vol. ethyl acetate. Ethyl acetate extract was evaporated to dryness and the lipid residue was subsequently resuspended in methanol. After extraction, the CCD tubules were homogenized and the protein concentration was measured. The samples were purified by

reverse phase (RP)-HPLC on a C_{18} μBondapak column (4.6 × 24 mm) using a linear gradient from acetonitrile:water:acetic acid (62.5:37.5:0.05%) to acetonitrile (100%) over 20 min at a flow rate of 1 ml/min. The fraction containing 11,12-EET was collected on the basis of the elution profile of standards monitored by ultraviolet absorbance (205 nm). The fractions were evaporated to dryness and resuspended in 100 μl of acetonitrile. HPLC fractions containing 11,12-EET were derivatized as described earlier(4). The derivatized 11,12-EET was dried with nitrogen and resuspended in 50 μl of iso-octane for gas chromatography-mass spectrometry (GC-MS) analyses. A 1 μl aliquot of derivatized CYP-derived AA metabolites, dissolved in iso-octane, was injected into a GC (Hewlett Packard 5890) column (DB-1ms; 10.0 m, 0.25 mm inner diameter, 0.25 μm film thickness, Agilent). We used temperature programs ranging from 150-300°C at rates of 25°C/min, respectively(16). Methane was used as a reagent gas at a flow resulting in a source pressure of 1.3 torr and the MS (Hewlett-Packard 5989A) was operated in electron capture chemical ionization mode. The endogenous 11,12-EET (ion m/z 319) was identified by comparison of GC retention times with authentic D_8 11,12-EET (m/z 327) standards.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Sources of Funding: The work was supported by NIH grant P01-HL34300 and P01-DK038266.

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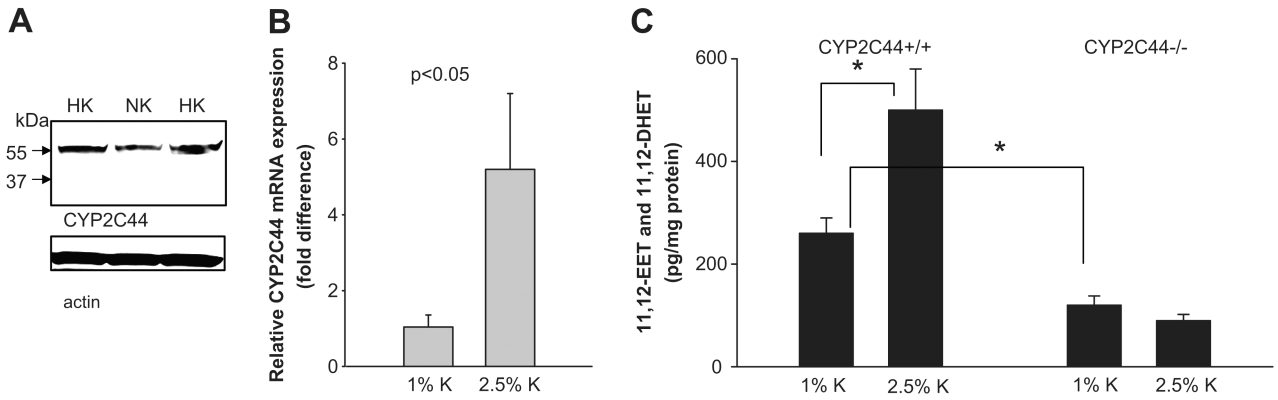

Fig. 1.

(A) A Western blot showing the effect of K diet on the expression of Cyp2c44 in the renal cortex and outer medulla (mixture) of wt mice on NK (1% KCl) or HK (2.5% KCl) for 7 days. (B) Effect of K diet on relative expression of Cyp2c44 mRNA in the CCD of wt mice on 1% K or 2.5% K diet for one week. (C) Effect of K diet on 11,12-EET and 11,12-DHET in the isolated CCDs of Cyp2c44(+/+) mice (wt) and of Cyp2c44(-/-) mice on a NK and a HK diet for one week. Asterisk indicates the significant difference between two groups.

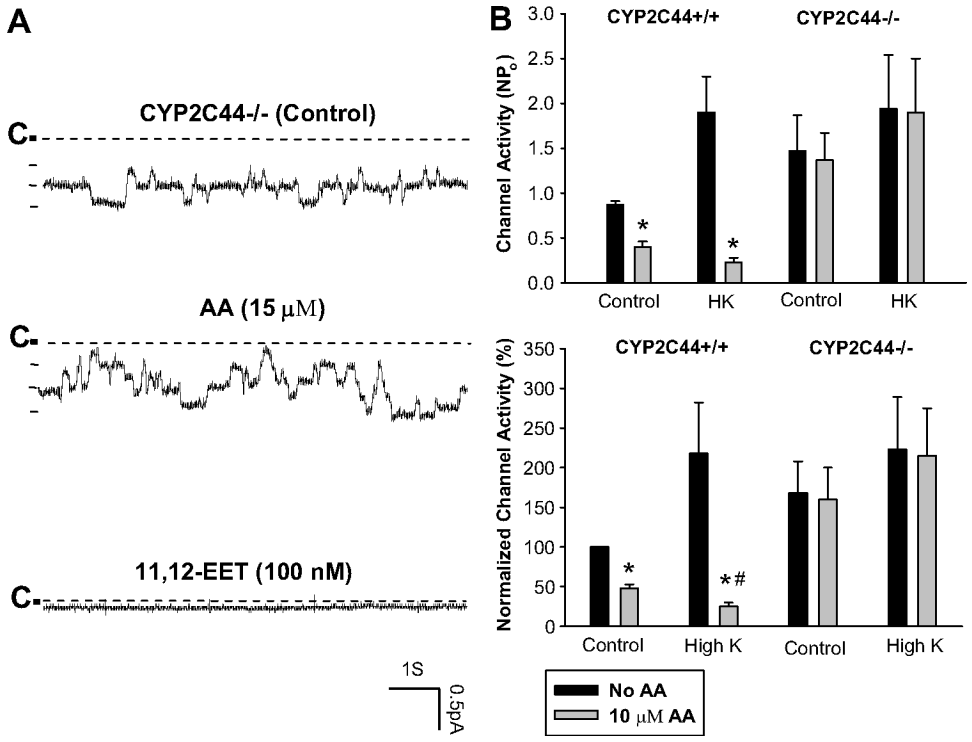

Fig.2.

(A) A single channel recording showing the effect of AA and 11,12-EET on ENaC in the CCD of Cyp2c44(-/-) mice on a HK diet for 3-7 days. The experiment was performed in a cell-attached patch and the holding potential was 60 mV. The channel closed level is indicated by a dotted line and "c". (B) Effect of arachidonic acid (AA) on ENaC activity (NP_o) (top panel) or the normalized channel activity (low panel) in wt and Cyp2c44(-/-) mice. Experiments were performed in cell-attached patches in the split-open CCD of wt and Cyp2c44-/- mice. AA was directly added to the bath and the channel activity was normalized by comparing NP_o of wt mice on a control diet. "*" indicates significant difference between the experimental group and the corresponding control. "#" indicates that data were significantly different from the rest of groups.

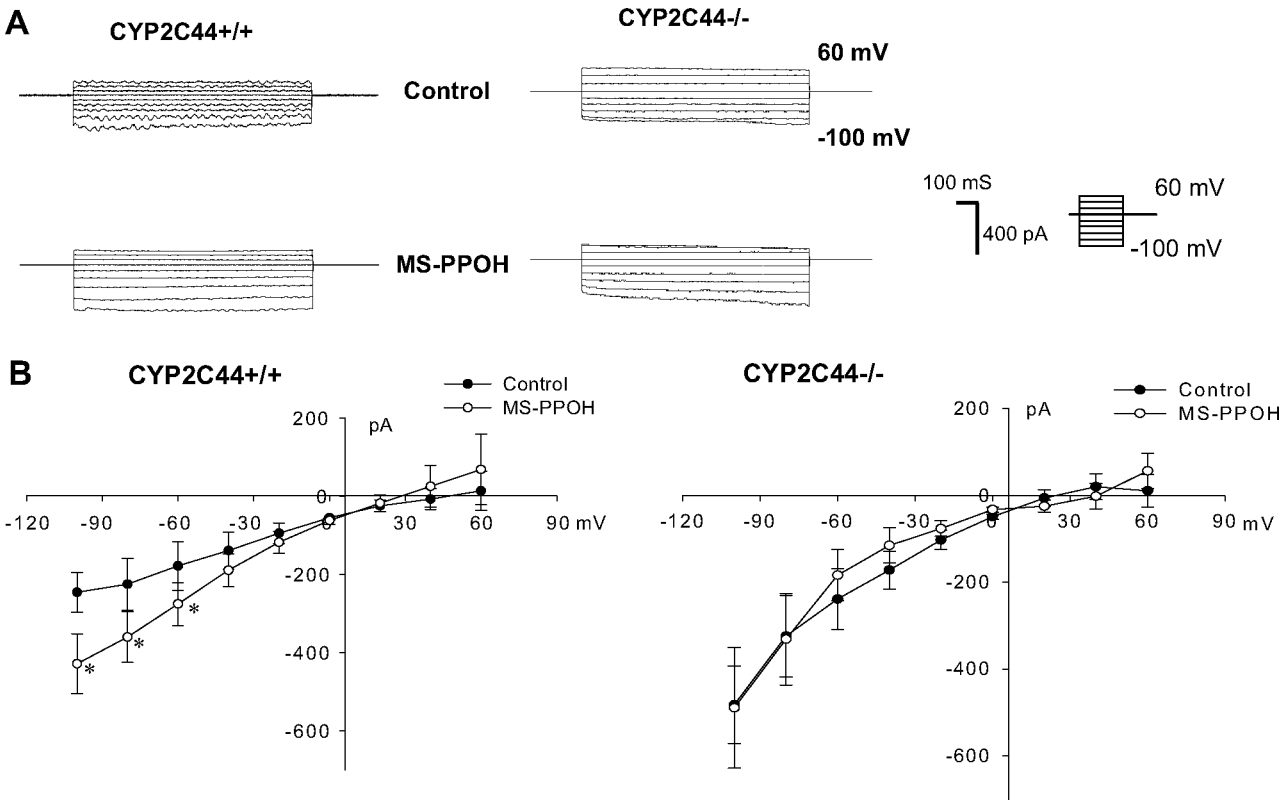

Fig. 3.

(A) The effect of MS-PPOH (5 μM) on the amiloride-sensitive whole-cell Na currents in principal cell of the CCD measured with the whole-cell recording at -100 mV to 60 mV with 20 mV step in wt or Cyp2c44(-/-) mice on a high K diet. The trace was subtracted amiloride-sensitive Na currents. (B) An I/V curve demonstrating the effect of inhibiting CYP epoxygenase on the whole-cell amiloride-sensitive Na currents in principal cell of the CCD in wt mice (left) and in Cyp2c44-/- mice (right) on a HK (2.5%) for one week. Asterisk indicates the significant difference in comparison to the corresponding controls.

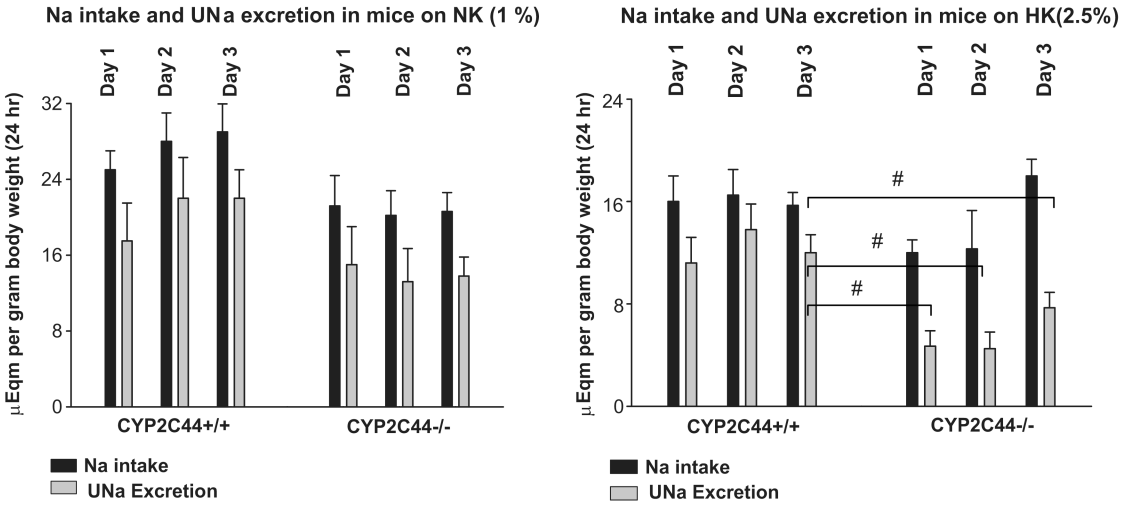

Fig.4.

Dietary Na intake and urinary Na excretion (UNa) in 24 hr in wt and Cyp2c44-/- mice on a NK or on a HK diet. Two mice were placed in a metabolic cage and 24 hr food intake and urinary Na excretion were recorded. The mice were kept on a NK diet for three days before switching to a HK diet for additional three days. Thus, UNa of the mice on NK diet at day 3 is served as the value at day zero for the mice on a high K diet. The significance was determined by comparing UNa of the *Cyp2c44(-/-)* mice with those of wt mice on a high K diet for three days. Note that different scales were used for presenting data from NK and HK's group. "#" indicates the significant difference between two groups.

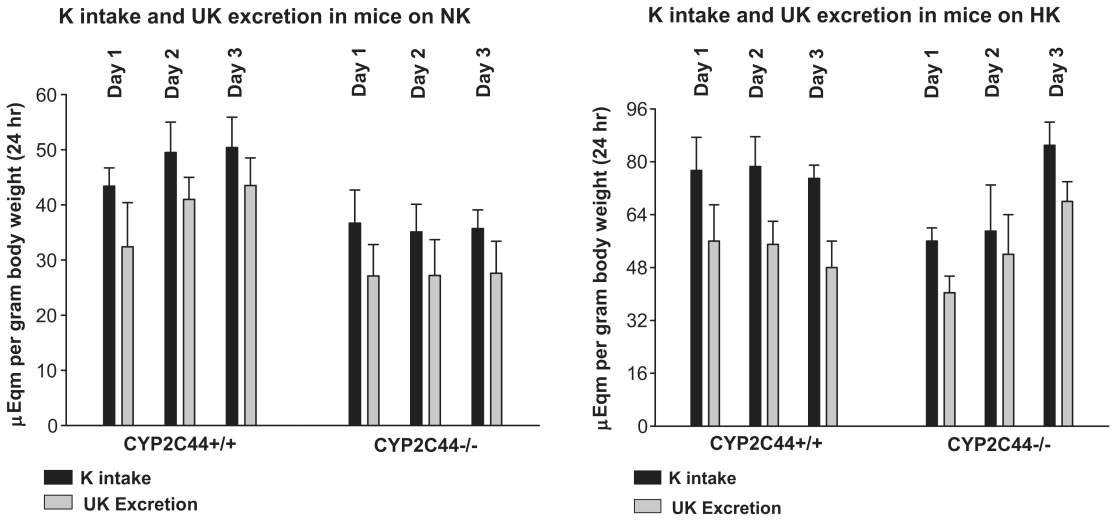

Fig.5.

Dietary K intake and urinary K excretion (UK) in 24 hr in wt and Cyp2c44(-/-) mice on a NK or on a HK diet. Two mice were placed in a metabolic cage and 24 hr food intake and urinary K excretion were recorded. The mice were kept on a NK diet for three days before switching to a HK diet for additional three days. Thus, UK of the mice on NK diet (1% K +0.3% Na) in day 3 is served as the value at day zero for the mice on a high K diet. Note that different scales were used for presenting data from NK and HK's group.

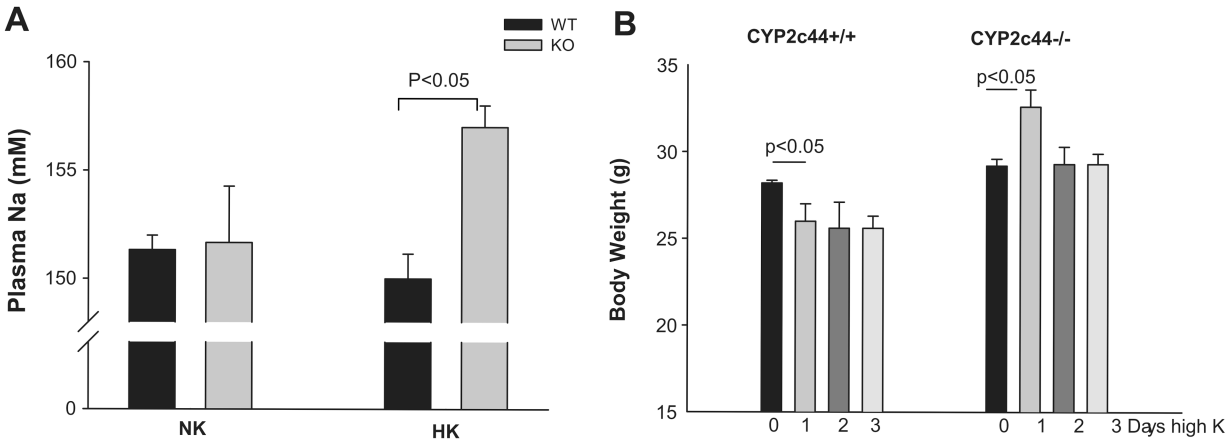

Fig. 6.

(A) Effect of dietary K intake on the plasma Na concentration in the wt and Cyp2c44(-/-) mice (KO). (B) Effect of increasing K intake from 1% to 2.5% on body weight of the wt and Cyp2c44(-/-) mice (KO).

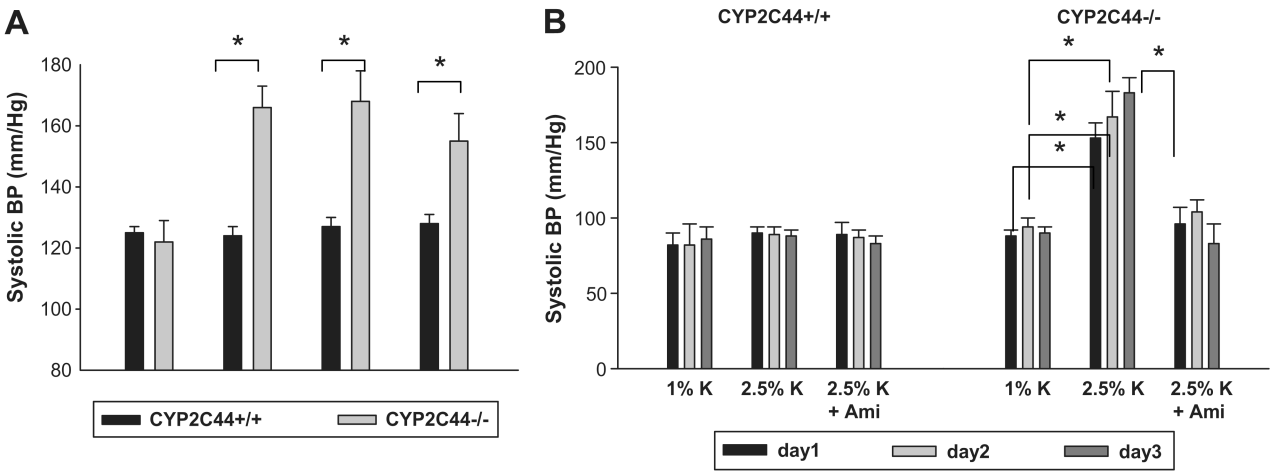

Fig. 7.

(A) Effect of increasing K intake on systolic blood pressure (BP) in the wt and Cyp2c44(-/-) mice. Mice were fed with normal salt (0.3% NaCl/ 1% KCl) or a HK diet (0.3% NaCl /2.5% KCl). The BP was measured with carotid artery catheter (N=8). (B) Effect of a high K intake on the blood pressure of wt and CYP2C44-/- mice in the absence of or in the presence of amiloride (0.5 mg/100g body weight) which was administered through drinking water. The BP was measured with tail-cuff methods and the value represents a mean value of measurements obtained from 3-5 mice. Asterisk indicates the significant difference between two groups.