Cyp2c44 Epooxygenase Is Essential for Preventing the Renal Sodium Absorption During Increasing Dietary Potassium Intake

Peng Sun, Joseph Antoun, Dao-Hong Lin, Peng Yue, Katherine H. Gotlinger, Jorge Capdevila, Wen-Hui Wang

Abstract—The aim of this study is to test whether the Cyp2c44 epooxygenase-dependent metabolism of arachidonic acid prevents the hypertensive effect of a high K (HK) intake by inhibiting the epithelial sodium channel (ENaC) activity. A HK intake elevated Cyp2c44 mRNA expression and 11,12-epoxyeicosatrienoic acid levels in the cortical collecting duct in Cyp2c44(+/+) mice (wild-type [wt]). However, an HK intake failed to increase 11,12-epoxyeicosatrienoic acid formation in the cortical collecting ducts of Cyp2c44(−/−) mice. Moreover, increasing K intake enhanced arachidonic acid–induced inhibition of ENaC in the wt but not in Cyp2c44(−/−) mice. In contrast, 11,12-epoxyeicosatrienoic acid, a Cyp2c44 metabolite, inhibited ENaC in the wt and Cyp2c44(−/−) mice. The notion that Cyp2c44 is the epooxygenase responsible for mediating the inhibitory effects of arachidonic acid on ENaC is further suggested by the observation that inhibiting Cyp-epooxygenase increased the whole-cell Na currents in principal cells of wt but not in Cyp2c44(−/−) mice. Feeding mice with an 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-hour urinary Na excretion and increasing Na concentration, and the effects were absent in wt mice. In contrast, disruption of the Cyp2c44 gene did not alter K excretion. We conclude that Cyp2c44 epooxygenase mediates the inhibitory effect of arachidonic acid on ENaC and that Cyp2c44 functions as an HK-inducible antihypertensive enzyme responsible for inhibiting ENaC activity and Na absorption in the aldosterone-sensitive distal nephron. (Hypertension. 2012;59:339-347.) Online Data Supplement

Key Words: 11,12-EET ■ arachidonic acid ■ ENaC ■ hypertension ■ kidney

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 Dietary Approaches to Stop Hypertension diet recommends all healthy adults to double their daily K intake to prevent salt-sensitive hypertension. However, the underlying mechanisms by which a high K (HK) intake prevents the salt-sensitive hypertension are not completely understood. Factors such as kallikrein4 and prostaglandins5 have been suggested to play a role in decreasing blood pressure (BP) and increasing renal Na excretion. Our previous study demonstrated that an HK intake stimulated the kidney expression of the rat Cyp2c23 epooxygenase, a homologue of the murine Cyp2c44 epooxygenase.6 A large body of evidence indicates that CYP2C epooxygenase–dependent arachidonic acid (AA) metabolism plays an important role in preventing the salt-sensitive hypertension.7-12 Inhibition of renal CYP2C epooxygenases caused hypertension in rats on high-Na diets.7,8 However, the animals became normotensive after the removal of the epooxygenase inhibitor, even when the animals were kept on a high-Na diet. The role of the epooxygenase in preventing salt-sensitive hypertension was best demonstrated in the Cyp4a10(−/−) mouse model.12 Although Cyp4a10 does not metabolize AA to epoxyeicosatrienoic acids (EETs),6 disruption of the Cyp4a10 gene reduced the expression of epooxygenase, such as Cyp2c44, and resulted in salt-sensitive hypertension.11 However, the identity of the antihypertensive epooxygenase isoform(s) is not unambiguously determined. Because Cyp2c44 homologues are epooxygenase isoforms responsible for 11,12-EET biosynthesis in the kidney7,9,13,14 and their expression is upregulated by an HK intake,15 we tested whether the Cyp2c44-dependent metabolism of AA plays a role in mediating the antihypertensive effects of an HK intake.

Methods

Animals and Metabolic Cage

Isogenic Cyp2c44(−/−) and Cyp2c44(+/+) mice were obtained from J.C. (see the supplemental Methods section for details). We
also purchased 129SvE mice (black Agouti coat), a strain of the 129SvJ mice from Taconic Farms (Germantown, NY), isogenic with the animals from Vanderbilt University. Mice were fed solid diets containing normal (1.0% KCl) or a high K (2.5% KCl). Disruption of Cyp2c44 reduced total EET/dihydroxyeicosatrienoic acid (DHET) excretion in the urine. However, the ratio among 8,9-, 11,12-, and 12,13-EET/DHET was similar between Cyp2c44+/- and Cyp2c44+-/- mice (please see Figure S1, available in the online Data Supplement at http://hyper.ahajournals.org/). Moreover, Western blot analysis demonstrated that expression of Cyp2c29, Cyp4a10, Cyp4a12, and Cyp4a14 in the kidney was similar in wild-type (wt) and Cyp2c44--/- mice (please see Figure S2).

We followed the protocol for the metabolic cage study described previously.16 After 3 days of cage training, mice were fed with normal diet (1.0% K+0.3%Na) for 3 days and an HK diet (2.5% K+0.3%Na) for additional 3 days. Their 24-hour food intake, urine output, and body weight were recorded daily. Urinary Na and K excretion in the urine. However, the ratio among 8,9-, 11,12-, and 12,13-EET/DHET was similar between Cyp2c44+/- and Cyp2c44+-/- mice (please see Figure S1, available in the online Data Supplement at http://hyper.ahajournals.org/). Moreover, Western blot analysis demonstrated that expression of Cyp2c29, Cyp4a10, Cyp4a12, and Cyp4a14 in the kidney was similar in wild-type (wt) and Cyp2c44--/- mice (please see Figure S2).

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Patch-Clamp Technique and Western Blot
We followed the protocol published previously by Frindt et al17 for the whole-cell patch-clamp experiments. The protocol for Western blot is described in the supplemental Methods section. To increase the possibility of finding epithelial sodium channel (ENaC), we used an Na-deficient (1.000% K with <0.001% Na) diet for 3 days as control. We demonstrated previously that the expressions of CYP2C44 and EET level in the cortical collecting duct (CCD) were similar in the animals on a low-Na diet for 3 days as those on a normal diet.15 For whole-cell recording, control mice were kept on a normal rat chow (1.0% K+0.3% Na).

Real-Time PCR to Detect Cyp2c44 Expression in the CCD
The RNA of the isolated CCD was isolated with RNAqueous-Micro kit (Ambion). The cDNA was generated with Affinity Script RT enzyme from Stratagene (La Jolla, CA). Briefly, 1 µL of random primer and 100 ng of RNA or single tubule lysates were annealed at 95°C by adding 1 µL of dithiothreitol, 2’-deoxyribonucleoside 5’-triphosphate, and enzyme. The mixture was incubated for 1 hour at 65°C. Before conducting the single-tubule PCR, the RT-PCR was performed with the mRNA purified from the kidney as template. The Cyp2c44 primers (2.5 nmol/L; sense, 5’-TTATCTCTTGGCC TGTGCTCC-3’; antisense, 5’-GGCCACACACGGAGTTCAC-3’) were mixed with 2.0 µL of cDNA (200 ng) and 12.5 µL of 2X SYBR Green master. MxPro3000 (Stratagene) was used for the real-time PCR experiments, and we used 2-ΔΔCT to analyze the comparative expression level of Cyp2c44. GAPDH was used as a control to normalize the expression of Cyp2c44.

Measurement of EET/DHET
We followed the method described previously to measure 11,12-EET/DHET.18 All of the solvents were high-performance liquid chromatography grade or better, and synthetic standards were used to obtain standard curves (5–500 pg) for each eicosanoid (linear regression R2 values >0.99) and internal standard (also see the online Data Supplement).

Measurement of BP
We measured the BP using a carotid artery catheter or noninvasive tail-cuff methods (Kent Scientific, Torrington, CT) between 9:00 and 11:00 AM. For inserting catheter, mice were anesthetized with Nembutal (75 mg/kg of body weight), and the carotid artery was separated from the vagus nerve and muscle. After cutting open the carotid artery, we inserted a polyethylene (PE-10) catheter into the vessel. The catheters were connected to a remote pressure sensor. After the animals became familiar with the environment, their BPs were measured with a Digi-Med Blood Pressure Analyzer (MicroMed Inc, Louisville, KY). The BP of each animal was monitored continuously for ≥40 minutes at an ambient temperature of 23°C, and ≥20 such measurements were performed to get the mean value. For tail-cuff method, mice were trained for 1 to 2 weeks before the start of experiments. Systolic BP measurements were recorded after 5 cycles of acclimatization, and 7 to 15 measurements were taken as the representative pressure for each mouse.

Solution and Statistics
AA and 11,12-EET were purchased from Cayman Chemicals (Ann Arbor, MI). The antibody for Cyp2c44 was generated from J.C. The data are presented as mean±SEM. We used either Student t test or 1-way ANOVA test to determine the statistical significance. P values ≤0.05 were considered significant.

Results
We demonstrated previously 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 expres-
The CYP epoxygenases metabolize AA to 5,6-, 8,9-, and 14,15- EET, and the 11,12-EET accounts for 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 an HK intake on 11,12-EET biosynthesis of the CCD in response to an HK intake.

Because 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. 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 NPo of wt mice on a control diet. *Significant difference between the experimental group and the corresponding control. #Data were significantly different from the rest of the groups.

These results identify Cyp2c44 as the epoxygenase responsible for the increases in 11,12-EET biosynthesis of the CCD in response to an HK intake.

Figure A. A single channel recording showing the effect of arachidonic acid (AA) and 11,12-EET on the epithelial sodium channel (ENaC) in the cortical collecting duct (CCD) of Cyp2c44(−/−) mice on a high-K (HK) diet for 3 to 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 AA on ENaC activity (NPo; a product of channel number (N) and open probability (Po)) in wild-type (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 NPo of wt mice on a control diet. *Significant difference between the experimental group and the corresponding control. #Data were significantly different from the rest of the groups.

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<th>No AA</th>
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N=4). These results identify Cyp2c44 as the epoxygenase responsible for the increases in 11,12-EET biosynthesis of the CCD in response to an HK intake.
findings are consistent with the previous report that AA-mediated inhibition of ENaC was enhanced in the CCD of rats on a 10% K diet.\textsuperscript{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\textsuperscript{(--/-)} mice on an HK diet for 3 to 7 days. We suspect that inhibition of CYP epoxygenase might increase ENaC activity if Cyp2c44 is involved in regulating channel basal activity. Figure 3A is a whole-cell recording showing that treatment of principal cells with N-methylsulfonyl-6-(2-propargyloxyphenyl)hexanamide (5 \textsuperscript{μ}mol/L), an inhibitor of CYP epoxygenase,\textsuperscript{21} increased amiloride-sensitive Na currents in principal cells from 246±50 pA (wt mice) to 482±90 pA (\textit{Cyp2c44}\textsuperscript{(--/-)} mice). Furthermore, inhibition of CYP epoxygenase with N-methylsulfonyl-6-(2-propargyloxyphenyl) hexanamide failed to increase Na currents in Cyp2c44\textsuperscript{(--/-)} mice (489±140 pA at −100 mV). Similar results were observed in the mice fed a normal diet (1.0% K+0.3% Na; please see Figure S3). Inhibition of epoxygenase increased whole-cell Na currents in wt mice but had no effect in Cyp2c44\textsuperscript{(--/-)} mice. Therefore, results strongly suggest that Cyp2c44-dependent AA metabolism is responsible for suppressing ENaC activity in principal cells of the wt mice fed an HK diet.

Because ENaC activity is upregulated in the CCD from Cyp2c44\textsuperscript{(--/-)} mice on an 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 the disrupted Cyp2c44 gene. Thus, we measured the food intake and urinary excretion of Na and K over a 24-hour period with metabolic cages in the wt and Cyp2c44\textsuperscript{(--/-)} mice fed a normal K diet for 3 days and then fed an HK diet for an additional 3 days. Results summarized in Figure 4 show the 24-hour dietary Na intake and urinary Na excretion (UNa) in the wt and Cyp2c44\textsuperscript{(--/-)} mice on a normal K diet (Figure 4, left) and on an HK diet (Figure 4, right). It is apparent that the ratios between UNa and dietary Na intake were not significantly different between wt and Cyp2c44\textsuperscript{(--/-)} mice.
fed a normal K diet for 3 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 an HK diet, UNa for Cyp2c44(-/-) mice was significantly lower than that of wt animals. The ratio for day 3 between UNa and Na intake for 24 hours decreased from 0.68 ± 0.04 (control diet) to 0.43 ± 0.04 (HK diet; N=8) in Cyp2C44(-/-) mice, whereas the ration was unchanged in the wt animals on an HK diet (0.76 ± 0.04). Therefore, lack of a functional Cyp2c44 epoxypgenase 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. Figure 5 summarizes experiments in which 24-hour dietary K intake and 24-hour urinary K excretion were examined in wt (N=8) and in Cyp2c44(-/-) mice (N=8) on a normal K or on an HK diet. From inspection of Figure 5, it is apparent that disruption of the Cyp2c44 gene did not significantly affect renal ability of K secretion under both control conditions and during an HK intake. The ratios between 24-hour urinary K excretion and K intake in mice on an 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 K or an HK diet for 3 days. As shown in Figure 6A, plasma Na concentrations were not significantly different for wt and Cyp2c44(−/−) mice fed a control K diet (wt: 151 ± 1 mmol/L; Cyp2c44[−/−]: 151.2 ± 2.5 mmol/L). However, when fed an HK diet, plasma Na levels were significantly higher in Cyp2c44(−/−) mice than those of wt mice (150 ± 1 and 156.9 ± 1 mmol/L, for wt and Cyp2c44[−/−] mice, respectively; N = 5). As a consequence of high plasma Na concentration and low UNa, Cyp2c44[−/−] mice gained weight from 28.2 ± 0.2 to 26.2 ± 1.1 g (P < 0.05; N = 4), whereas, in contrast, the wt animals lost weight from 29.2 ± 0.2 to 26.6 ± 0.7 g (P < 0.05; N = 4) 1 day after animals were fed with an HK diet (Figure 6B). The above results suggest that an 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 with the wt mice. Although the aldosterone levels were similar under control condition (wt: 550 ± 50 pg/mL; Cyp2c44[−/−]: 450 ± 50 pg/mL), an HK intake for 3 days increased the plasma aldosterone in wt mice (1500 ± 300 pg/mL), a value that was significantly higher than those in the Cyp2c44(−/−) animals (900 ± 200 pg/mL; N = 4).

To determine whether a decrease in UNa caused hyper-tension in Cyp2c44(−/−) mice, we measured on a daily basis the BP of wt and Cyp2c44(−/−) mice fed diets containing either normal (1.0% KCl) or HK (2.5% KCl) from 1 to 3 days. When fed a normal K, the systolic BPs of wt and Cyp2c44(−/−) mice were not significantly different (125 ± 4 and 122 ± 7 mm Hg for wt and Cyp2c44[−/−] mice, respectively; Figure 7A). However, within the first 24 hours of exposure to an HK diet, the BP of Cyp2c44[−/−] mice increased markedly (to 166 ± 7 mm Hg; N = 8) and remained high as long as the mice were fed with the HK diet (Figures 7A and 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 (Figure 7A and S4). Thus, increasing dietary K intake causes hypertension only in Cyp2c44(−/−) mice.

Figure 7. A, Effect of increasing K intake on systolic blood pressure (BP) in the wild-type (wt) and Cyp2c44(−/−) mice. Mice were fed with normal salt (0.3% NaCl/1.0% KCl) or a high-K (HK) diet (0.3% NaCl/2.5% KCl). The BP was measured with carotid artery catheter (N=8). B, Effect of an HK intake on the BP of wt and CYP2C44(−/−) mice in the absence of or in the presence of amiloride (0.5 mg/100 g of 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 to 5 mice. *Significant difference between 2 groups.
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 an HK diet on the BP in Cyp2c44(−/−) mice (Figure 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.25–27 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.28 We have demonstrated previously that CYP-epoxygenase−dependent AA metabolites inhibited ENaC activity in the CCD15,18,26 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 that developed hypertension when fed a high-salt diet and have impaired regulatory control of their Cyp2c44 epoxygenase by dietary salt.15 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: patch-clamp experiments demonstrated that AA failed to inhibit, whereas 11,12-EET was able to block ENaC in the CCD of Cyp4a10(−/−) mice, and 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 raising the BP in animals with downregulated epoxygenase activity.7 Another finding of the present study is 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 an HK intake stimulates the expression of Cyp2c44 in the kidney, especially in the CCD.15 Moreover, the effect of an HK intake on Cyp2c44 was specific, because an HK intake failed to increase CYP2J2 expression, which is expressed in the CCD.20 The observation that an 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 an 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 aldosterone-sensitive distal nephron (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 attributed to a decrease in Na-Cl cotransport expression in the distal convoluted tubule,32 thereby reducing Na absorption in the distal convoluted tubule. 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 3 lines of evidence: (1) increasing dietary K content from 1.0% to 2.5% raises the BP in Cyp2c44(−/−) mice but not in the wt mice; (2) the 24-hours UNa was lower in Cyp2c44(−/−) mice on an HK diet than those of wt animals on an HK diet; (3) plasma Na concentrations and body weight were significantly higher in Cyp2c44(−/−) mice on an HK diet than those of the wt mice. We hypothesize that an HK intake specifically stimulates Cyp2c44 expression in the ASDN, including CCD (please see Figure S5). Increased Cyp2c44 expression enhances AA metabolism to generate 11,12-EET, which inhibits ENaC. Therefore, we propose that, although an 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 that a mechanism other than stimulation of ENaC activity may also contribute to the HK intake−induced increase in BP in Cyp2c44(−/−) mice. It has been reported that an 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 BP in animals with downregulated epoxygenase activity.

Another finding of the present study is that Cyp2c44 disruption did not affect the renal ability to excrete K, because the ratios between 24-hour urinary K secretion and dietary K intake in Cyp2c44(−/−) mice were similar to those wt mice under control conditions and during an HK adaptation. We have demonstrated previously that 11,12-EET activates the Ca2+−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,
an HK intake is known to stimulate renal outer medullary potassium channel expression,\textsuperscript{32,34–36} thereby compensating for the function of BK channels. Relevant to this proposal was the report that renal K secretion was not compromised in BK channel knockout mice.\textsuperscript{37} Second, increased ENaC activity in Cyp2c44 (−/−) mice augments the driving force for K secretion through renal outer medullary potassium 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 renal outer medullary potassium 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 medullary potassium channel activity and enhanced driving force for K secretion.

**Perspectives**

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

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**Disclosures**

None.

**References**


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

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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 x 5mm cover-glass coated with polylysine and then transferred 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 NP0, was calculated from data samples of 60 seconds duration in the steady state as follows:

\[ NP_0 = \sum (t_1 + 2t_2 + \ldots + it_i) \]

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 MgCl₂, 1.8 CaCl₂ and 5 HEPES (pH=7.4). The bath solution for single channel patch-clamp experiments contained (in mM) 135 NaCl, 5 KCl, 1.8 CaCl₂, 1.8 MgCl₂, 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 CaCl₂, 1 MgCl₂, 2 glucose, 5 mM BaCl₂, and 10 HEPES adjusted to pH 7.4 with NaOH. The tip of the pipette was filled with pipette solution followed by back-filling 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-BS 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 (Na₃VO₄) (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 µg/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 D₈ 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 C₁₈ μBondapak column (4.6 X 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 D₈ 11,12-EET (m/z 327) standards.
**Results**

Fig. S1 The urinary epoxygenase excretion (EETs+DHETs) in Cyp2C44+/+ and Cyp244/-/- mice (left panel) (N=4). The bar graph in the right panel shows the ratio among 14,15-, 11,12-, and 8,9-EET/DHET in Cyp2C44+/+ and Cyp244/-/- mice.
Fig.S2  A Western blot shows the expression of CYP4a10, Cyp4a12, Cyp4a14, Cyp2c29 and actin in the kidneys from Cyp2c44+/+ (wt) and Cyp2c44-/- mice (KO), respectively.
Fig. S3 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 in wt or Cyp2c44(-/-) mice.
Fig. S4  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). Asterisk indicates a significant difference from the corresponding control value.
High Potassium Intake

Aldosterone

CYP Epoxygenase 2C44

11,12-EET

ENaC expression

ENaC

Renal Na Excretion

Fig. S5 A scheme illustrates the mechanism by which increasing dietary K intake regulates ENaC activity and Na excretion in the collecting duct.