High Potassium Intake Enhances the Inhibitory Effect of 11,12-EET on ENaC

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ABSTRACT

High dietary potassium stimulates the renal expression of cytochrome P450 (CYP) epoxygenase 2C23, which metabolizes arachidonic acid (AA). Because the AA metabolite 11,12-epoxyeicosatrienoic acid (11,12-EET) can inhibit the epithelial sodium channel (ENaC) in the cortical collecting duct, we tested whether dietary potassium modulates ENaC function. High dietary potassium increased 11,12-EET in the isolated cortical collecting duct, an effect mimicked by inhibiting the angiotensin II type I receptor with valsartan. In patch-clamp experiments, a high potassium intake or treatment with valsartan enhanced AA-induced inhibition of ENaC, an effect mediated by a CYP-epoxygenase–dependent pathway. Moreover, high dietary potassium and valsartan each augmented the inhibitory effect of 11,12-EET on ENaC. Liquid chromatography/mass spectrometry showed that the rate of EET conversion to dihydroxyeicosatrienoic acids (DHET) was lower in renal tissue obtained from rats on a high-potassium diet than from those on a control diet, but this was not a result of altered expression of soluble epoxide hydrolase (sEH). Instead, suppression of sEH activity seemed to be responsible for the 11,12-EET–mediated enhanced inhibition of ENaC in animals on a high-potassium diet. Patch-clamp experiments demonstrated that 11,12-DHET was a weak inhibitor of ENaC compared with 11,12-EET, whereas 8,9- and 14,15-DHET were not. Furthermore, inhibition of sEH enhanced the 11,12-EET–induced inhibition of ENaC similar to high dietary potassium. In conclusion, high dietary potassium enhances the inhibitory effect of AA and 11,12-EET on ENaC by increasing CYP epoxygenase activity and decreasing sEH activity, respectively.


We previously demonstrated that cytochrome P450 (CYP) epoxygenase-dependent arachidonic acid (AA) metabolism inhibited epithelial sodium channel (ENaC) in the cortical collecting duct (CCD) and that 11,12-epoxyeicosatrienoic acid (11,12-EET) was responsible for mediating the effect of AA on ENaC.1 Furthermore, the observation that AA failed to inhibit ENaC in the CCD of the mice with a low expression of CYP2C44 suggests that CYP2C44 and its orthologs may be responsible for mediating the inhibitory effect of AA.2 The expression of CYP2C44 or its orthologs has been shown to be regulated by dietary Na intake: A high Na intake stimulates3 whereas a low Na intake suppresses the expression of CYP2C44 homologue.3 A large body of evidence has suggested that EET plays a role in the regulation of renal Na transport and salt-sensitive hypertension.1,2,4,5 Inhibition of CYP epoxygenase-dependent AA metabolism results in the development of salt-sensitive hypertension5,6; however, the BP returned to normal after the removal of the epoxygenase inhibitor, even when the animals were still kept on a high-Na diet. The high Na intake–induced in-

Received November 4, 2009. Accepted May 13, 2010. Published online ahead of print. Publication date available at www.jasn.org.

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crease in EET formation was defective in CYP4A10(−/−) mice. Although CYP4A10 is not the enzyme responsible for generating EET, deleting the CYP4A10 gene impairs the expression of CYP epoxygenases, CYP2C44 in particular. Consequently, CYP4A10(−/−) mice had developed a salt-sensitive hypertension that was prevented by amiloride, suggesting that defective regulation of ENaC by CYP epoxygenase-dependent AA metabolism was responsible for the salt-sensitive hypertension in CYP4A(−/−) mice. The expression of CYP2C44 or its ortholog is stimulated not only by high Na but also by high potassium (HK) intake. Because CYP2C44 is highly expressed in the connecting tubule and the CCD, it is conceivable that a high expression of CYP2C44 should enhance the AA-induced inhibition of ENaC; therefore, the aim of this study was to examine whether HK intake enhances the CYP-epoxygenase-dependent inhibitory effect of AA on ENaC.

RESULTS

We previously demonstrated that HK intake stimulates the expression of CYP2C23 in the renal cortex and outer medulla (OM) in the rat kidney. In this study, we reexamined the effect of HK intake on the CYP2C23 expression in the renal cortex and OM. Because alteration of dietary K and Na intake has the same effect on CYP2C23 expression in the renal cortex and OM (data not shown), we used the tissue mixture dissected from renal cortex and OM for the experiments. Figure 1A is a

Figure 1. HK intake stimulates the expression of rat CYP2C23. (A) Western blot showing the effect of 10% K diet on CYP2C23 in the cortex and OM of rat kidney. (B) 11,12-EET levels in the CCD of rats on a control, 10% HK (7 days), low Na (3 days), and valsartan treatment + low Na (3 days). *Significant difference in comparison with control. (C) Channel recording showing the effect of 100 nM 11S,12R- or 11R,12S-EET on ENaC. The experiments are performed in a cell-attached patch, and the holding potential was 60 mV. Results are summarized in a bar graph shown in the bottom of the figure.
Western blot showing that HK intake (10%) increased the CYP2C23 expression in the rat kidney by 100 ± 10% (n = 4 from three animals). The effect of HK intake on CYP2C23 was not the result of increasing aldosterone level because low Na intake for 3 days had no significant effect on CYP2C23 (Figure 1A), whereas prolonged low Na intake (14 days) decreased the expression of CYP2C23 in the rat kidney.3 We also measured 11,12-EET, a major EET in the kidney,5 in isolated CCD tubules. Figure 1B summarizes the results demonstrating that HK intake significantly increased the 11,12-EET level in the rat CCD from 0.75 ± 0.13 (control) to 1.65 ± 0.30 pg/μg protein (four rats). In contrast, low Na intake for 3 days had no significant effect on 11,12-EET generation (0.70 ± 0.12 pg/μg protein), suggesting that aldosterone is not responsible for the HK-induced stimulation of CYP epoxygenase activity.

It has been reported that rat CYP2C23 or mouse CYP2C44 is able to metabolize AA to either 11S,12R-EET or 11R,12S-EET, which is the principal AA metabolite generated by CYP2C23 (rat) or CYP2C44 (mouse).8 Thus, we used patch-clamp technique to examine the effect of 11S,12R- or 11R,12S-EET on ENaC in the CCD. Figure 1C is a typical channel recording made in a cell-attached patch demonstrating that application of 100 nM 11R,12S-EET (directly adding EET into the bath) completely inhibited ENaC (n = 5). Moreover, 11R,12S-EET had no effect on ENaC in inside-out patches (data not shown), suggesting that the effect of 11R,12S-EET on ENaC is indirect and may require the involvement of the intracellular signaling molecules. In contrast, application of 100 nM 11S,12R-EET had no effect on Na channels (n = 5); therefore, results suggest that HK intake stimulates the expression of CYP2C23 in the rat kidney and that the major CYP2C23-dependent AA metabolite, 11R,12S-EET, mediates the effect of AA on ENaC.

**HK Intake Enhances AA and 11,12-EET-Mediated Inhibition of ENaC**

Because HK intake stimulates the expression of CYP2C23 in the rat kidney and increases the 11,12-EET level in the CCD, we expect that AA-mediated inhibition of ENaC should be enhanced in the CCD from animals on an HK diet; therefore, we examined the effect of AA on ENaC in the CCD of rats on an HK diet for 7 days and a low Na intake for 3 days or for 14 days, respectively. Because ENaC activity in the CCD from rats on a normal-Na diet is low, it is technically difficult to detect the ENaC activity in the CCD of animals on a normal-Na diet. We therefore used the CCD from rats on a low-Na diet for 3 days as the control. As shown in Figure 1A, the expression of CYP2C23 in the renal cortex and OM of rats on low-Na diet for 3 days was only slightly lower than those on a normal rat diet. Figure 2A is a channel recording showing that the application of 15 μM AA inhibited ENaC by 75 ± 5% (n = 6) in the CCD of rats on a low-Na diet for 3 days; however, only 5 μM AA was required to achieve the same level of inhibition of ENaC (n = 5) in the CCD from rats on an HK diet (Figure 2B). Data summarized in Figure 2C from five experiments demonstrate that the EC50 value of AA was approximately 3 μM (7-day HK) whereas it increased to 7 to 8 μM (3-day low Na) and >15 μM (14-day low Na), respectively. This suggests that an HK intake enhances whereas a low-Na intake diminishes the AA-mediated inhibition of ENaC.

HK intake not only increases the sensitivity of ENaC to AA but also enhances the 11,12-EET–induced inhibition of ENaC. Figure 3A is a channel recording showing that 100 nM 11,12-EET blocked ENaC activity by 90 ± 10% (n = 6) in the CCD of rats on an HK diet for 7 days, whereas 200 nM 11,12-EET was required to achieve a similar level of the inhibition in mice on a low-Na diet for 3 days (n = 6). Figure 3B is a dose-response curve of the 11,12-EET effect on ENaC in rats on an HK diet for 7 days, a low-Na diet for 3 days, and a low-Na diet for 14 days. It is apparent that the EC50 value for 11,12-EET in the HK-fed animals was approximately 50 nM, which was lower than those on a low-Na diet (100 nM). Moreover, the potency of 11,12-EET–mediated inhibition of ENaC was not affected by the length of low-Na intake because EC50 was the same in the CCD from rats on a low-Na diet for 3 or 14 days. Thus, the results demonstrate that HK intake significantly enhances both AA- and 11,12-EET–mediated inhibition of ENaC in the CCD.

**Inhibiting of Angiotensin II Type I Receptor Mimics the Effect of a HK Intake**

HK intake is known to suppress renin-angiotensin II (AngII) signaling.9,10 Thus, it is possible that inhibition of the AngII signaling pathway may be involved in upregulation of CYP2C23. To test this hypothesis, we examined the expression of AngII type I receptor (AT1R) in the kidney (mixture of renal cortex and OM) of rats on a normal-K (1%), or an HK diet (2.5 and 10.0%). Figure 4A is a representative Western blot from four such experiments (three rats). It is apparent that an increase in dietary K intake suppressed the expression of AT1R by 50 ± 6% (2.5% K) and 75 ± 10% (10%), respectively. If diminishing AngII-dependent pathway is responsible for mediating the effect of HK intake on CYP2C23 expression, then inhibition of AT1R should mimic the effect of HK intake and increase CYP2C23 expression. Hence, we treated rats with valsartan for 3 days while maintaining animals on a low-Na diet and examined the effect of valsartan treatment on CYP2C23 expression in the kidney 3 days after the treatment. From the inspection of Figure 4B, it is apparent that inhibiting AT1R increased the expression of CYP2C23 by 90 ± 12% (n = 4 from three rats). We also measured the 11,12-EET level in the isolated CCD from valsartan-treated rats on a low-Na diet for 3 days. Figure 1B summarizes the results of four experiments (four rats) showing that valsartan treatment mimicked the effect of HK intake and increased 11,12-EET level in the CCD (1.8 ± 0.3 pg/μg protein; four rats).

We next examined the effect of AA and 11,12-EET on ENaC in the CCD from valsartan-treated rats on low Na for 3 days (Figure 5). Data summarized in Figure 5A show that inhibition of AT1R significantly enhanced the inhibitory effect of AA on
ENaC. EC$_{50}$ value of AA was approximately 3 μM (valsartan) which was similar to those on an HK diet, although the rats were still maintained on a low-Na diet. This is in sharp contrast to the results obtained from nontreated rats on a low-Na diet, which increased EC$_{50}$ value of AA-induced inhibition to 7 to 8 μM (Figure 2C). Also, data summarized in Figure 5B demonstrate that inhibition of AT1R mimicked the effect of HK intake and enhanced the 11,12-EET–mediated inhibition of ENaC in valsartan-treated rats. Inhibiting AT1R decreased EC$_{50}$ value of 11,12-EET–induced inhibition of ENaC from approximately 100 nM (Figure 3C) to 50 nM, which is the same as those in animals on an HK diet (Figure 5B). Hence, the results strongly suggest that the HK intake–induced enhancement of AA- and 11,12-EET–induced inhibition of ENaC is the result of inhibiting AT1R.

HK Intake Decreases the Activity of Soluble Epoxide Hydrolase
Stimulation of the AngII pathway has been reported to upregulate soluble epoxide hydrolase (sEH) in vascular endothelium.$^{11}$ Because sEH is involved in converting EET to DHET, $^{12,13}$ we suspect that the HK intake–induced enhancement of 11,12-EET–mediated inhibition may be the result of suppressing sEH activity; therefore, we examined the expression of sEH and the enzymatic activity by measuring the rate of 11,12-EET conversion to 11,12-DHET in the tissue dissected from renal cortex and OM of rats on a normal-K and an HK diet for 7 days. Figure 6A is a representative Western blot from four experiments demonstrating that HK intake did not alter the expression of sEH compared with those on a normal-K diet; however, the activity of sEH was significantly lower in rats on an HK diet than those on a normal-K diet. Figure 6B summarizes the results of four experiments demonstrating that the rate of EET conversion to DHET in the kidney from rats on an HK diet was significantly lower (0.05 ± 0.01 pM/min per mg protein) than those on a normal-K diet (0.11 ± 0.01 pM/min per mg protein). The notion that low sEH activity may be responsible for the HK-induced enhancement of 11,12-EET–mediated inhibition of ENaC was also supported by the

Figure 2. HK intake enhances the AA-induced inhibition of ENaC. (A and B) A single-channel recording demonstrating the effect of AA on ENaC in rats on a low-Na (A) or on an HK diet (B). The channel closed level is indicated by “C” and a dotted line. The experiments are performed in a cell-attached patch with a holding potential of 60 mV. (C) Dose-response curve of the AA-induced inhibition of ENaC in the CCD of rats on different diets. Each data point represents four to six experiments. *Significant difference in comparison with the corresponding control value (low Na for 3 days). Data are normalized by taking channel activity (NPo) under control conditions (in the absence of AA) as 100%. 

finding that 11,12-DHET had a weak inhibitory effect on ENaC. Figure 7A is a channel recording demonstrating that application of 100 nM 11,12-DHET slightly inhibited ENaC activity, whereas the same concentration of 11,12-EET almost completely blocked ENaC (Figure 3). Data summarized in Figure 7B show that 100 nM 11,12-DHET decreased channel activity (NPo) of ENaC from 2.3 ± 0.5 to 1.5 ± 0.4 (n = 7) in the CCD of rats on an HK diet, whereas 8,9- and 14,15-EET had no effect on ENaC. Thus, results indicate that 11,12-DHET is a weak inhibitor of ENaC.

sEH Is Involved in Regulating ENaC in Rats with HK Intake

If the HK-induced augmentation of 11,12-EET–mediated inhibition of ENaC is the result of suppressing sEH activity, then inhibition of sEH should enhance the 11,12-EET–induced inhibition of ENaC and abolish the different response of ENaC to 11,12-EET in animals on an HK diet and a low-Na diet. Hence, we examined the effect of 11,12-EET on ENaC in the CCD treated with 100 nM 12-(3-adamantan-1-yl-ureido) dodecanedioic acid (AUDA), a selective sEH inhibitor. Figure 8A is a single-channel recording showing that application of 50 nM 11,12-EET inhibited ENaC by 95 ± 5% (n = 6) in the CCD from rats on an HK diet for 7 days (Figure 8A, top) and on a low-Na diet for 3 days (Figure 8A, bottom). Moreover, in the presence of AUDA, the EC50 value of 11,12-EET–induced inhibition of ENaC is the same (20 nM) in rats on a HK diet for 7 days or on a low-Na diet for 3 days (Figure 8B). The results suggest that suppression of sEH activity may be
responsible for the HK-induced enhancement of 11,12-EET–mediated inhibition of ENaC.

**DISCUSSION**

The major enzymes responsible for the AA metabolism in the kidney include cyclooxygenase (COX), lipoxygenase, and CYP monooxygenase; however, a large body of evidence indicates that both COX-dependent and CYP enzyme–dependent metabolites of AA play an important role in the regulation of membrane transport in the kidney. CYP epoxygenase converts AA to EETs such as 5,6, 8,9, 11,12, and 14,15. Moreover, 11,12-EET accounts for >60% of the total renal EET. EET has been reported to diminish the effect of vasopressin on water permeability in the CCD and to activate the Ca2+-dependent big-conductance K channel in the CCD. CYP-epoxygenase–dependent AA metabolism also plays a role in mediating big-conductance K channel–dependent and flow-stimulated K secretion. Application of 5,6-EET has been shown to decrease Na absorption by inhibiting the Na/H exchanger in the rabbit CCD. Because indomethacin abolished the effect of 5,6-EET on Na transport, it was suggested that the effect of 5,6-EET on the Na/H exchanger in the CCD may be mediated by a COX-dependent pathway. We previously demonstrated that 11,12-EET inhibits ENaC in the CCD, suggesting that CYP epoxygenase plays an important role in regulating renal Na transport.

CYP epoxygenases responsible for metabolizing AA to EET in the rat kidney are CYP2C11, 2C12, 2C23, 2C24, and 2J family, whereas in the mouse kidney, they are 2C29, 2C38, 2C39, 2C44, and 2J5. CYP2C23 has been shown to be the major homologue of CYP epoxygenase in the rat kidney and is mainly responsible for forming 11,12-EET. Mouse CYP2C44 is an ortholog of rat CYP2C23 and shares 84% identical amino acid sequence. We previously showed that CYP2C44 is expressed in the CCD and that the expression is upregulated by either high-Na intake or HK intake. The recombinant CYP2C44 expressed in Escherichia coli was able to metabolize AA to EETs. Moreover, the major product generated by CYP2C44-dependent AA metabolism is 11R,12S-EET. In this regard, we have observed that 11R,12S-EET is a potent inhibitor of ENaC, whereas 11S,12R-EET at the same concentration failed to inhibit ENaC, suggesting 11R,12S-EET is responsible for mediating the effect of AA on ENaC.

The first finding of this study is that HK intake enhanced the AA-induced inhibition of ENaC. The possibility that inhibition of AngII signaling pathway by HK intake is involved in upregulation of CYP2C23 is supported by the observations that valsartan treatment mimicked the effect of HK intake and increased the expression of CYP2C23 in the kidney and 11,12-EET level in the isolated CCD. Relevant to our observation is...
the report that AngII decreased transcription and translation of several CYP epoxygenases, including CYP2C44 in the kidney.22,23 Because a high expression of CYP2C23 is expected to increase the conversion of AA to 11,12-EET, it is conceivable that the AA-induced inhibition of ENaC should be enhanced in the CCD from rats on an HK diet and in valsartan-treated rats. This notion is supported by the observation that the EC50 value of AA-mediated inhibition of ENaC is smaller in animals on an HK diet or valsartan-treated rats than those on a low-Na diet. Although the observation that valsartan treatment mimics the effect of HK intake supports the role of AT1R, the direct evidence demonstrating that AngII and AT1R are involved in mediating the effect of HK intake on CYP2C23 is not clearly established. Moreover, HK intake or valsartan treatment could directly or indirectly enhance AT2R function, which may be involved in stimulating CYP epoxygenase activity; therefore, we need further experiments to provide direct evidence demonstrating the role of AT1R in mediating the effect of HK intake on 11,12-EET generation and on the AA-induced inhibition of ENaC.

The second major finding of this study is that HK intake augmented the 11,12-EET–induced inhibition of ENaC. The observation that valsartan treatment enhanced 11,12-EET–induced inhibition of ENaC further supports the notion that HK-induced suppression of AT1R is involved in augmenting the effect of 11,12-EET on ENaC. Two lines of evidence suggest that HK intake–induced enhancement of 11,12-EET–mediated inhibition of ENaC was the result of suppressing sEH activity: (1) The metabolic rate of EET to DHET was significantly lower in the renal tissue from rats on an HK diet than those on a normal-K diet; (2) inhibiting sEH with AUDA not only increased the sensitivity of ENaC to 11,12-EET but also abolished the difference in EC50 values between HK-fed and control animals. Because sEH is responsible for metabolizing 11,12-EET to 11,12-DHET,13 it is conceivable that suppression of sEH activity induced by HK intake should effectively increase the availability of 11,12-EET, thereby enhancing the inhibitory effect of 11,12-EET. This notion is also supported by the observation that 11,12-DHET is a weak inhibitor of ENaC in comparison with 11,12-EET.

The mechanism by which HK intake inhibits sEH activity is not clear. AngII has been shown to stimulate sEH expression in endothelial cells and in the myocytes.11,24 Furthermore, AngII-induced stimulation of sEH was achieved at both transcription and translation levels by a mechanism involving activating AP-1, a transcription factor; however, the observation that HK intake did not change the sEH protein expression in the kidney does not support the possibility that decreased sEH protein expression was responsible for low enzymatic activity of sEH in the kidney of rats on an

Figure 7. 11,12-DHET is a weak inhibitor of ENaC. (A) A single-channel recording in a cell-attached patch showing the effect of 100 nM 11,12-DHET on ENaC in the rat CCD. The experiments are performed in a cell-attached patch with a holding potential of 60 mV. (B) Bar graph summarizes the effect of 100 nM 8,9-, 14,15-, and 11,12-DHET on ENaC activity in the rat CCD. Each data point represents four to six measurements. *Significant difference in comparison with the control.
HK diet. Therefore, the HK intake–induced suppression of sEH activity is likely the result of posttranslational regulation. Figure 9 is a scheme illustrating a possible mechanism by which HK intake enhances the AA-mediated inhibition of ENaC. We propose that HK intake suppresses the AngII signaling pathway, thereby inhibiting AT1R. Suppression of AT1R decreases AngII-induced inhibition of CYP2C23 and diminishes the AngII-mediated stimulation of sEH activity. Consequently, high CYP2C23 activity and low sEH activity augment 11,12-EET–induced inhibition of ENaC.

The physiologic importance of this finding may partially explain the observation that HK intake suppresses renal Na excretion despite that HK intake stimulates the aldosterone level, which is expected to increase renal Na absorption.25–27 The underlying mechanism by which HK intake enhances urinary Na excretion is not completely understood. Factors such as kallikrein28 and prostaglandins29 have been suggested to play a role in increasing renal Na excretion. In addition, downregulation of renin–AngII pathway and decreasing superoxide anion generation are two important factors that may be involved in lowering BP. Moreover, HK intake has been shown to decrease the expression of Na-Cl co-transporter in the distal nephron, thereby decreasing Na absorption.30 HK intake has been shown to increase ENaC activity in the isolated split-open CCD31; however, it is possible that 11,12-EET–induced inhibition of ENaC attenuates Na absorption in the collecting duct during HK intake despite high ENaC channel expression in the plasma membrane. Because Na transport through ENaC provides a critical driving force for K secretion, inhibition of ENaC by 11,12-EET is expected to decrease the driving force for K secretion in the CCD in animals on an HK diet, which is known to stimulate K secretion in the collecting duct; however, this

Figure 8. Inhibition of sEH enhances the inhibitory effect of 11,12-EET. (A) A single-channel recording in a cell-attached patch showing the effect of 50 nM 11,12-EET on ENaC in the rat CCD of animals on an HK diet (top) and a low-Na diet (bottom). The experiments are performed in a cell-attached patch with a holding potential of 60 mV. The channel closed level is indicated by “C” and dotted lines. (B) Dose-response curve of 11,12-EET–induced inhibition of ENaC in AUDA-treated and untreated CCD. Each data point represents four to six measurements. *Value measured in animals on an HK diet is significantly different from the corresponding values measured in animals treated with AUDA. The data are normalized by taking channel activity (NPo) under control conditions (no 11,12-EET) as 100%.

Figure 9. A scheme illustrating a possible mechanism by which HK intake enhances the AA- and 11,12-EET–induced inhibition of ENaC.
apparent paradox may be solved by the possibility that 11,12-EET might activate the transient receptor potential (TRP) channels in the CCD, thereby increasing the driving force for K secretion. TRP channels have been shown to be expressed in the collecting duct. Moreover, CYP epoxygenase–dependent metabolites including 11,12-EET have been shown to open TRP channels. In this regard, we have identified a 40-pS nonselective cation channel in the apical membrane of the CCD (W.-H.W, unpublished data, 2010). Moreover, the cation channels are activated by luminal application of 11,12-EET and inhibited by gadolinium. Because further characterization of the EET-sensitive cation channels is beyond the scope of this study, data regarding EET-sensitive cation channels will be presented in a separate study. We conclude that HK intake stimulates the expression of CYP2C23 and decreases the activity of sEH and that high CYP2C23 and low sEH activity are responsible for enhancing AA-induced and 11,12-EET–mediated inhibition of ENaC during HK intake. We speculate that the HK-induced enhancement of AA-mediated inhibition of ENaC is partially involved in increasing renal Na excretion by HK intake.

CONCISE METHODS

Preparation of CCDs
Male Sprague-Dawley rats (5 to 6 weeks, <90 g) were used in the experiments and were fed with control diet containing 1% K or an HK diet (10%). The control diet contained 0.4% Na, 1.18% K, 0.95% Ca\(^{2+}\), 0.66% phosphorus, 0.67% Cl, 0.21% Mg\(^{2+}\), and 13.5% fat (AA <0.01%; LabDiet, Brentwood, MO) and the HK diet contained 0.4% Na, 10% KCl, 1% Ca\(^{2+}\), 0.6% phosphorus, 0.16% Mg\(^{2+}\), and 18.1% fat (no detectable AA; Harlan Laboratory, Madison, WI). Animals were killed by cervical dislocation, and kidneys were removed immediately. Several thin slices of the kidney (<1 mm) were cut and placed on an ice-cold Ringer solution, and the CCD was isolated. For immobilization of the tubules, they were placed on a 5 × 5-mm cover glass coated with polylysine and then transfused to a chamber (1000 μl) mounted on an inverted Nikon microscope. The CCDs were superfused with HEPES-buffered NaCl solution. The CCD was cut open with a sharpened micropipette to expose the apical membrane. For studying the effect of inhibiting AT1R on AA- and 11,12-EET–induced inhibition of ENaC, we treated rats with valsartan (40 mg/kg per d) or losartan using gavage; the control rats were fed with solvent. Because no functional difference between losartan and valsartan was observed, data are pooled.

Patch-Clamp Technique
An Axon2008 patch-clamp amplifier was used to record channel current. The currents were low-pass filtered at 50 Hz by an eight-pole Bessel filter (902LPF; Frequency Devices, Haverhill, MA) and digitized by an Axon interface (Digidata 1300). Data were analyzed using the pClamp software system 9 (Axon). Channel activity, defined as NPo, was calculated from data samples of 60 seconds’ duration in the steady state as follows:

\[ \text{NPo} = \sum(t_1 + 2t_2 + \ldots + t_i) \]

where \(t_i\) is the fractional open time spent at each of the observed current levels. The pipette solution for studying Na channels contained (in mM) 135 NaCl, 5 KCl, 1.8 MgCl\(_2\), 1.8 CaCl\(_2\), and 5 HEPES (pH 7.4). Both AA and 11,12-EET were directly added to the bath to reach their final concentrations. Also, when we compared the channel activity in animals on different diet or treatment, we normalized data by taking the initial control values (without AA or 11,12-EET) as 100%. Otherwise, channel activity is presented as real NPo.

Tissue Preparation and Western Blot
Five to six rats were used for each set of experiments. The renal cortex and OM were separated under a dissecting microscope and suspended in RIPA solution (1:8 ratio, wt/vol) containing 50 mM Tris–HCl (pH 7.4), 10 mM NaCl, 1% NP-40, 0.1% Triton X-100, 0.1% SDS, 1 mM sodium molybdate, 1 mM para-nitrophenyl-phosphate, and 1 mM EDTA. For every 125-μg tissue sample, we added a 25-μl cocktail of protease and phosphatase inhibitors containing aprotinin (1 μg/ml), leupeptin (1 μg/ml), pepstatin A (1 μg/ml), sodium vanadate (Na\(_3\)VO\(_4\); 1.5 mM), and sodium fluoride (1 mM). The samples were left on ice for 15 minutes 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 minutes. The mixture was then centrifuged at 3000 rpm for 10 minutes at 4°C and the resultant supernatant collected. Moreover, after performing electrophoresis, we stained the gel with 0.25% Coomassie blue R-250 to confirm the equal load.

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-buffed saline (TBS), rinsed, and washed with 0.05% Tween20-TBS buffer. An Odyssey infrared imaging system (LI-COR, Lincoln, NE) was used to scan the membrane at wavelength of 680 or 800 nM.

Measurement of EET
Isolated CCDs were homogenized on ice in 1 ml of methanol, and 500 pg of d\(_8\)-11,12-EET was added as internal standard. Samples were stored at −80°C for >30 minutes before centrifugation for 15 minutes at 4°C. Supernatants were collected, diluted with water, and acidified to pH 4.0 with 2 M HCl. The pellet was saved and used for protein determination using Bio-Rad Protein Assay (cat. no. 500-006). C18-ODS AccuBond II 500-μg cartridges (Agilent Technologies, Santa Clara, CA) were primed first with 20 ml of methanol followed by 20 ml of water. Samples were loaded, washed with 20 ml of water and 6 ml of hexane, and eluted with 6 ml of methanol. The collected methanol fraction was dried under nitrogen, resuspended in 200 μl of methanol, and stored at −80°C until analysis by liquid chromatography tandem mass spectrometry (LC/MS/MS). All solvents were HPLC grade or better.

LC/MS/MS
Eicosanoid identification and quantification was performed with a Q-trap 3200 linear ion trap quadrupole LC/MS/MS equipped with a Turbo V ion source operated in negative electrospray mode (Applied
Biosystems, Foster City, CA). Extracted samples were suspended in 10 μl of methanol and injected into the HPLC via an Agilent 1200 standard series autosampler equipped with a thermostat set at 4°C (Agilent Technologies). The HPLC component consisted of an Agilent 1100 series binary gradient pump equipped with an Agilent Eclipse plus column (Agilent Technologies). The HPLC component consisted of an Agilent 1100 series autosampler equipped with a thermostat set at 4°C (Agilent Technologies). The column was eluted at a flow rate of 0.500 ml/min with 100% mobile phase A (methanol/water/acetic acid (60:40:0.01, vol/vol/vol)) from 0 to 2 minutes and a gradient increasing to 100% B (100% methanol) at 13 minutes. Multiple reaction monitoring was used with a dwell time of 25 or 50 ms for each compound, with source parameters as follows: Ion spray voltage, −4500 V; curtain gas, 40 U; ion source gas flow rate 1, 65 U; on source gas flow rate 2, 50 U; and temperature, 600°C. Synthetic standards were used to obtain standard curves (5 to 500 pg) for each eicosanoid (linear regression R² > 0.99) and internal standard.

**Assay for sEH Activity**

For determination of hydrolysis of EETs in the renal CCD, 14,15-, 11,12-, and 8,9-EET (6 ng each) were added to the tubule suspension (2-ml volume) in PBS at 37°C for 5 minutes with shaking around a 3-mm orbit at 600 rpm in a VWR Incubating Mini Shaker. EET and DHET extraction for electrospray ionization (ESI)/LC/MS analyses were performed as described previously. In brief, a Finnigan LCQ Advantage quadrupole ion-trap MS (Thermo Fisher Scientific) equipped with ESI source run by Xcalibur software was used. Reversed-phase HPLC was run with a Luna C18(2) 250 × 2.0-mm column (Phenomenex, Torrance, CA) maintained at 30°C with an isocratic eluent of acetonitrile/water/methanol/acetic acid (60:30:10:0.05) at a flow rate of 0.30 ml/min. For EET hydrolysis kinetic studies, the isocratic eluent was maintained for 5 minutes and followed by a gradient to a final composition of acetonitrile/water/methanol/acetic acid (75:15:10:0.05) in 15 minutes. ESI was carried out at an ion transfer tube temperature of 260°C, a spray voltage of 4.5 kV, a sheath gas flow of 34 U, and an auxiliary gas flow of 20 U (units refer to arbitrary values set by the LCQ software). MS/MS breakdown for m/z 337 was at an energy level of 30% by the instrument, and a 7-point Gaussian smoothing was applied in the mass data processing.

**Statistical Analysis**

The bath solution for patch-clamp experiments contained (in mM) 140 NaCl, 5 KCl, 1.8 CaCl₂, 1.8 MgCl₂, and 10 HEPES (pH 7.4). AA, DHET, and 11,12- and 8,9-EET were purchased from Santa Cruz Biotechnology (University of California at Davis) and Dr. Jorge Capdevila (University of Vanderbilt, Ann Arbor, MI). AUDA, an inhibitor of sEH, was synthesized in Dr. Capdevila’s laboratory (Southwestern Medical Center at Dallas). The antibodies for sEH and CYP2C23 were a gift from Dr. B.D. Hammock (University of California at Davis) and Dr. Jorge Capdevila (University of Vanderbilt), respectively. The AT1R antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The data are presented as means ± SEM. We used one-way ANOVA test to determine the statistical significance. P < 0.05 was considered to be significant.

**ACKNOWLEDGMENTS**

The work is supported by National Institutes of Health grants HL34300 and GM31278 and the Robert A. Welch Foundation.

**DISCLOSURES**

None.

**REFERENCES**


