Effects of cytochrome P-450 metabolites of arachidonic acid on the epithelial sodium channel (ENaC)

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1Department of Physiology and 2Kidney Disease Center, Medical College of Wisconsin, Milwaukee, Wisconsin; 3Institute of Cytology, Russian Academy of Sciences, St. Petersburg, Russian Federation; and 4Department of Pharmacology and Toxicology, University of Mississippi Medical Center, Jackson, Mississippi

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Pavlov TS, Ilavskaya DV, Levchenko V, Mattson DL, Roman RJ, Staruschenko A. Effects of cytochrome P-450 metabolites of arachidonic acid on the epithelial Na channel (ENaC). Am J Physiol Renal Physiol 2011;301:F672–F681, 2011. First published June 22, 2011; doi:10.1152/ajprenal.00597.2010.—Sodium reabsorption via the epithelial Na channel (ENaC) in the aldosterone-sensitive distal nephron plays a central role in the regulation of body fluid volume. Previous studies have indicated that arachidonic acid (AA) and its metabolite 11,12-EEt but not other regiosomers of EETs inhibit ENaC activity in the collecting duct. The goal of this study was to investigate the endogenous metabolism of AA in cultured mpkCCDc14 principal cells and the effects of these metabolites on ENaC activity. Liquid chromatography/mass spectrometry analysis of the mpkCCDc14 cells indicated that these cells produce prostaglandins, 8,9-EEt, 11,12-EEt, 14,15-EEt, 5-HETE, 12/8-HETE, and 15-HETE, but not 20-HETE. Single-channel patch-clamp experiments revealed that 8,9-EEt, 14,15-EEt, and 11,12-EEt all decrease ENaC activity. Neither 5-, 12-, nor 15-HETE had any effect on ENaC activity. Diclofenac and ibuprofen, inhibitors of cyclooxygenase, decreased transepithelial Na+ transport in the mpkCCDc14 cells. Inhibition of cytochrome P-450 (CYP450) with MS-PPOH activated ENaC-mediated sodium transport when cells were pretreated with AA and diclofenac. Coexpression of CYP2C8, but not CYP4A10, with ENaC in Chinese hamster ovary cells significantly decreased ENaC activity in whole-cell experiments, whereas 11,12-EEt mimicked this effect. Thus both endogenously formed EETs and their exogenous application decrease ENaC activity. Downregulation of ENaC activity by overexpression of CYP2C8 was PKA dependent and was prevented by myristoylated PKI treatment. Biotinylation experiments and single-channel analysis revealed that long-term treatment with 11,12-EEt and overexpression of CYP2C8 decreased the number of channels in the membrane. In contrast, the acute inhibitory effects are mediated by a decrease in the open probability of the ENaC. We conclude that 11,12-EEt, 8,9-EEt, and 14,15-EEt are endogenously formed eicosanoids that modulate ENaC activity in the collecting duct.

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Factors regulate the renal handling of sodium, including the renin-angiotensin-aldosterone system, nitric oxide, arachidonic acid (AA), and its metabolites. AA is primarily metabolized by cytochrome P-450 enzymes to 19- and 20-HETE and EETs (5,6-, 8,9-, 11,12-, and 14,15-EET) in the renal cortex, and these compounds have been shown to play critical roles in the regulation of renal tubular and vascular function (34).

Cytochrome P-450 members of the CYP2C and CYP4A families are the predominant epoxygenases and ω-hydroxylation enzymes expressed in the kidney (5). The Cyp4a10 gene in mice produces a salt-sensitive form of hypertension that is associated with alterations in the activity of ENaC (28). Similarly, Cyp2C isomers are expressed in the CCD (22, 46, 48), and abnormalities in the formation of EETs have been linked to the development of hypertension in Dahl salt-sensitive rats (23). AA and its CYP metabolites have multiple effects on ion channels (26). It was previously shown that AA significantly decreases ENaC activity in freshly isolated rat CCDs (46, 53). Moreover, it was proposed that adenosine inhibits ENaC activity by stimulation of the A1 adenosine receptor in the CCD, and the effect of adenosine is mediated by an increase in the formation of 11,12-EET (54). Recently, Sun et al. (47) demonstrated that high dietary potassium enhances the inhibitory effect of AA and 11,12-EET on ENaC. 11,12-EET has also been shown to mediate AA-induced inhibition of 18-Ps basolateral K+ channels (51) and activation of Ca2+-sensitive BK potassium channels in the apical membrane of the CCD (48). 20-HETE and EETs have effects on thick ascending limb (TAL) cells that decrease sodium reabsorption. The inhibitory action of 20-HETE on Na+ transport in the TAL is associated with closure of the apical 70-Ps K+ channels (10, 50). AA also inhibits the 50-Ps K+ channels in the basolateral membrane of the mTAL mainly through cytochrome P-450-dependent metabolites of AA (11). Recently, it was demonstrated that AA inhibits the 10-Ps chloride channel in the basolateral membrane of the mTAL and this effect is mediated by 20-HETE (12). Thus AA and its metabolites clearly are involved in the regulation of various ion channels in the kidney.

Previous studies have indicated that cytochrome P-450 metabolites of AA are produced in the CCD and inhibit ENaC activity (46, 47, 53, 54). However, their exact distribution and precise mechanism of action remain to be determined. The goal of the present study was to investigate the endogenous metabolism of AA and the effect of these metabolites on the activity of ENaC channels in mpkCCDc14 principal cells and Chinese
hamster ovary (CHO) cells transiently transfected to express ENaC channels. These models gave us the opportunity to study signaling mechanisms in more detail than was previously done using freshly isolated CCD.

MATERIALS AND METHODS

cDNA constructs and cell culture. CHO cells were obtained from ATCC (Manassas, VA), maintained under standard culture conditions (DMEM, 10% FBS, 1× penicillin-streptomycin, 37°C, 5% CO2), and transfected using the Polyfect reagent (Qiagen, Valencia, CA) as described previously (38, 41). For expression of mouse ENaC (mENaC) in CHO cells, subunit cDNA transfection ratios of 1:1:1 were used with 0.3 g of each cDNA/35-mm culture dish. The plasmids encoding α-, β-, and γ-ENaC have been described previously (33, 40). To define successfully transfected cells, 0.5 g of cDNA encoding green fluorescent protein (GFP) was added to the cDNA mix. The Cyp4a10 cDNA was kindly provided by Dr. F. Park and described previously (29). The Cyp2c8 cDNA (SC107944) was purchased from OriGene (Rockville, MD).

Immortalized mouse cortical collecting duct (mpkCCDc14) principal cells were kindly provided by Dr. A. Vandewalle (INSERM, Paris, France) and grown in defined medium on permeable supports (Costar Transwells, 0.4-μm pore, 24-mm diameter) as described previously (2, 32, 42). The cells were maintained with FBS and corticosteroids, allowing them to polarize and form a monolayer with high resistance and avid Na+ reabsorption. The cells were seeded onto permeable supports at a density of 0.2–0.3 × 10⁶ cells/filter. The mpkCCDc14 cells were kept on filter supports for at least 7 days, and the medium was changed every second day. Growth medium was composed of equal volumes of DMEM and Ham’s F12, 60 mM sodium selenate, 5 μg/ml transferrin, 50 nM dexamethasone, 1 nM triiodothyronine, 10 ng/ml EGF, 5 μg/ml insulin, 2% FCS, and 100 μg/ml penicillin/streptomycin. The cells were grown in an incubator with a 5% CO2-95% air atmosphere at 37°C. Typically, after 7 days a confluent transporting cell monolayer developed which was subsequently assessed by recording short-circuit voltage and transepithelial resistance.

(±)-15-HETE (catalog no. 34700), (±)-12/8-HETE (catalog no. 34550), (±)-5-HETE (catalog no. 34210), and N-methylsulfonyl-6-(2-

Table 1. Level of arachidonic acid metabolites (pM/mg protein) detected by LC/MS in mpkCCDc14 cells under control conditions and after incubation with arachidonic acid

<table>
<thead>
<tr>
<th></th>
<th>PGE₂</th>
<th>PGF₂</th>
<th>PGD₂</th>
<th>15-HETE</th>
<th>12/8-HETE</th>
<th>5-HETE</th>
<th>14,15-EET</th>
<th>11,12-EET</th>
<th>8,9-EET</th>
</tr>
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<tbody>
<tr>
<td>Before</td>
<td>0.69±0.34</td>
<td>0.71±0.35</td>
<td>0.45±0.23</td>
<td>2.61±1.19</td>
<td>1.15±0.53</td>
<td>1.49±0.70</td>
<td>2.57±1.17</td>
<td>3.70±1.6</td>
<td>1.86±0.80</td>
</tr>
<tr>
<td>After</td>
<td>2.80±1.24</td>
<td>1.90±0.94</td>
<td>1.02±0.50</td>
<td>2.11±0.90</td>
<td>1.83±0.87</td>
<td>2.23±0.93</td>
<td>2.48±0.92</td>
<td>3.02±0.98</td>
<td>1.81±0.66</td>
</tr>
</tbody>
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Values are means ± SE; n = 3 for prostaglandins and n = 5 for HETEs and EETs. LC/MS, liquid chromatography/mass spectrometry. See MATERIALS AND METHODS for details.

Fig. 1. Liquid chromatography/mass spectrometry (LC/MS) profiling of metabolites of arachidonic acid produced by cultured mpkCCDc14 principal cells. mpkCCDc14 cells were plated, harvested, pelleted, and resuspended in serum-free medium as described in MATERIALS AND METHODS. Cells were analyzed either under control conditions (A) or after incubation with arachidonic acid (AA; 40 μM) and NADPH (1 nM) for 30 min (B).
propargyloxyphenyl) hexanamide (MS-PPOH; catalog no. 75770) were purchased from Cayman Chemical. (±) 5,6-Epoxyeicosa-8Z,11Z,14Z-trienoic acid (catalog no. BML-EE11), (±) 11,12-epoxyeicosa-5Z,8Z,14Z-trienoic acid (catalog no. BML-EE14), and PKI (14–22) amide (myristoylated, catalog no. BML-P210–0500) were obtained from Enzo Life Sciences.

**Electrophysiology.** Whole-cell macroscopic current recordings of mENaC expressed in CHO cells were made under voltage-clamp conditions using standard methods (17, 38, 40). The current through ENaC was the inward, amiloride-sensitive Na+ current measured in a bath solution containing (in mM) 160 NaCl, 1 CaCl2, 2 MgCl2, and 10 HEPES (pH 7.4) and a pipette solution containing (in mM) 120 CsCl, 5 NaCl, 5 EGTA, 2 MgCl2, 2 ATP, 0.1 GTP, and 10 HEPES (pH 7.4). Current recordings were acquired with an Axopatch 200B (Axon Instruments, Union City, CA) interfaced via a Digidata 1440 (Axon Instruments) to a PC running the pClamp 10.2 suite of software (Axon Instruments). All currents were filtered at 1 kHz. Whole-cell capacitance was routinely compensated and estimated as an average of ~8 pF for CHO cells. Series resistances, average 2–4 MΩ, were also compensated.

For cell-attached patches made on the plasma membrane of mpkCCDc14 or CHO cells, the bath and pipette solutions were (in mM) 155 NaCl, 1 CaCl2, 2 MgCl2, and 10 HEPES (pH 7.4) and 140 LiCl, 2 MgCl2 and 10 HEPES (pH 7.4), respectively. Patch potentials were clamped (−Vp) to −60 mV. Currents were low-pass filtered at 200 Hz by an eight-pole Bessel filter (Warner Instruments), digitized, and stored on a PC using the Digidata 1440 interface. Patches were selected for low noise and drift of the baseline current (low noise is associated with higher seal resistances). Slow baseline drifts were corrected for some cell-attached patches. To assess the steady-state probability of channel opening, patches were clamped to a potential of −60 mV, and control channel activity was measured for at least 1 min before application of the corresponding drugs. The first 10 s of recording after application of a voltage step were omitted from the steady-state analysis. An equilibration period of 5 min was allowed after applications of HETEs and EETs to achieve a new steady state of channel activity. The channel openings were analyzed by Clampfit 9.0 or 10.2 software using the single-channel search in the analyze function. A 50% threshold cross-method was utilized to determine valid channel openings. All events were carefully checked visually before being accepted. NPo, the product of the number of channels (N) and the open probability (Po), or Po itself, were used to measure the channel activity within a patch. Single-channel unitary current (i) was determined from the best-fit Gaussian distribution of amplitude histograms. Channel activity was assessed as NPo = II, where I is mean total current in a patch, and i is unitary current at this voltage. When multiple-channel events were observed in a patch, the total number of functional channels in the patch was determined by the peaks detected on all-point amplitude histograms.

A Millicel Electrical Resistance System (Millipore, Billerica, MA) was used to measure voltage and resistance across the mpkCCDc14 cell monolayers grown on permeable supports as described previously (18, 20, 32). Equivalent transepithelial Na+ currents were calculated...
as the quotient of transepithelial voltage to transepithelial resistance under short-circuit conditions.

**AA metabolite detection with liquid chromatography/mass spectrometry.** mpkCCDc14 cells were collected using 0.05% trypsin, pelleted, and resuspended in serum-free media. The suspension was incubated for 30 min at 37°C in the presence of 1 mM NADPH and a saturating concentration of AA (40 μM) or extracted without incubation with AA and NADPH. The reaction was stopped by acidification with formic acid to pH 3.5. The cells were homogenized by sonication and extracted twice with 3 ml of ethyl acetate after the addition of 2 ng of an internal standard (d6-20-HETE). The organic phase was dried under nitrogen. The metabolites of AA were separated by HPLC on a Betabasic C18 column (150 × 2.1 mm, 3 μm, Thermo Hypersil-Keystone, Bellefonte, PA) at a flow rate of 0.3 ml/min, using isocratic elution with a mixture of acetonitrile:methanol:water:acetic acid in the ratio 38.25:6.75:55:0.01 for 15 min, then 51:9:40:0.01 for 40 min followed by a step gradient to 68:13:19:0.01 for 15 min. The effluent was ionized using negative ion electrospray and peaks eluting with a mass/charge ratio (m/z) of 319 > 245 (20-HETE), 319 > 301 (HETEs and EETs), 325 > 251 (internal standard) or 351 > 271 (PGD2 and PGE2), and 353 > 309 (PGF2) were monitored using Applied Biosystems API 3000 liquid chromatography mass spectrometry (LC/MS) as described previously (1, 33a). The ratio of ion abundance in the peaks of interest vs. that seen in the internal standard was determined and compared with standard curves generated over a range from 0.2 to 10 ng for 20-HETE and from 1.0 to 10 ng for the other metabolites. To identify the profile of eicosanoids produced by CHO cells transfected with Cyp2C8, cells were seeded on 35-mm dishes and transfected with GFP (1 μg) or Cyp2c8 (1 μg). Cells with media or media alone were collected upon confluence. The lipid fraction was extracted with ethyl acetate as described above for measurement of eicosanoids. Cellular EET levels were calculated as the difference between the values in cells extracted with media vs. the values obtained in the media alone.

**Cell viability assay.** mpkCCDc14 cells were seeded onto 12-well cluster plates at a subconfluent levels and were allowed to grow for at least 1 wk to form a monolayer. A modified MTT assay was used to determine the percentage of viable cells as described previously (18, 32). Diclofenac (100 and 500 μM), ibuprofen (1 mM), or vehicle was added to the media, and the cells were incubated for 4 h. After treatment, 0.5 mg/ml MTT (Sigma-Aldrich) was added, and cells were incubated for additional 4 h. The medium was aspirated and replaced with isopropanol to solubilize the formazan products, and the optical density of the extract was measured at 570 nm. The cell viability as a percentage of the viable cells was calculated from the absorbance values.

**Membrane-labeling experiments.** Membrane-labeling experiments followed those described previously (16, 39). In brief, CHO cells were transfected with Myc-tagged ENaC subunits alone or together with Cyp2c8. Furthermore, CHO cells expressing ENaC subunits were treated with 11,12-EET (150 nM) for 10 min or 4 h. Twenty-four hours after transfection or 5 min after drug treatment, the cells were

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**Fig. 4.** HETEs do not affect ENaC activity in mpkCCDc14 cells. Continuous current traces from representative cell-attached patches in mpkCCDc14 cells before and after treatment with 5-HETE (A), 12/8-HETE (B), or 15-HETE (C). Areas before (I) and after (II) treatment are shown at the bottom with an expanded time scale. Summary graphs of experiments testing the acute effects of HETEs on ENaC NPo are shown on the right.
washed twice with ice-cold Ca²⁺ and Mg²⁺ containing PBS (pH 8.0) and subsequently incubated with 0.25 mg/ml EZ-Link Sulfu-NHS-SS-Biotin (21331, Thermo Scientific) for 40 min at 4°C in the dark. Biotinylation was quenched by washing cells with 100 mM glycine containing PBS. The cells were harvested by scraping them into a lysis buffer (GLB) containing 1.0% Nonidet P-40 and a protease inhibitor cocktail (Roche, Indianapolis, IN). Brief-pulse sonication was used on samples to ensure efficient lysis. After sonication, the cells were spun down for 3 min at 10,000 g, and total protein concentration of the supernatant was measured using the DC detergent tolerant protein assay (Bio-Rad, Hercules, CA). One milligram of supernatant protein was incubated with 150 nM of streptavidin-agarose beads (Pierce, Rockford, IL) for 1 h at 4°C. The agarose beads were then washed, and bound protein was eluted by boiling for 1 min in a reducing SDS sample buffer. The proteins were separated by electrophoresis on 7.5% polyacrylamide gels in the presence of SDS, transferred to nitrocellulose, and probed with anti-Myc antibody in Tris-buffered saline supplemented with 1% dry milk and 0.1% Tween 20.

Statistics. All data are reported as means ± SE. The significance of differences in mean values was compared using either Student’s paired t-test or one-way ANOVA (post hoc comparison with Bonferroni multiple correction). A P < 0.05 was considered to be significant.

RESULTS

Identification of CYP metabolites of AA in mpkCCDc14 cells. LC/MS analysis revealed the presence of 15-, 12/8-, and 5-HETEs and 14,15-, 11,12-, and 8,9-EETs, but not 20-HETE in the mpkCCDc14 cells both before (Fig. 1A) and after incubation with AA (40 μM; 30 min) (Fig. 1B). This analysis also demonstrated the presence of PGF₂α, PGE₂, and PGD₂ in these cells. Interestingly, incubation of the cells for 30 min with exogenous AA increased the levels of prostaglandins, whereas the levels of EETs and HETEs did not increase (Fig. 1B, Table 1).

EETs but not HETEs acutely decrease ENaC activity in mpkCCDc14 cells. These experiments were performed using polarized epithelial monolayers of the mpkCCDc14 cells with robust transepithelial transport and resistance. Grown on permeable supports, these cells developed a lumen negative transepithelial potential difference (Fig. 1A). Amiloride (10 μM) was continuously monitored in paired cell-attached patch-clamp experiments. Extracellular application of 150 nM 11,12-EET rapidly decreased ENaC activity. The representative patch in Fig. 2A (1 of 6), formed on the apical membrane of a polarized principal cell, was clamped with a −60-mV test potential and remained at least two ENaC. A continuous trace before and after addition of 11,12-EET is shown at the top. Segments before and after 11,12-EET are shown at expanded time scales. As summarized in Fig. 2B, 11,12-EET acutely decreased ENaC Nₚₒ within 5 min from 2.14 ± 0.85 to 0.56 ± 0.33 (n = 6).

The effects of other EETs and HETEs on ENaC activity in the mpkCCDc14 cells are presented in Figs. 3 and 4. 8,9-EET and 14,15-EET (150 nM) significantly decreased ENaC activity (Fig. 3). Nₚₒ was 0.79 ± 0.08 before and 0.32 ± 0.08 after application of 150 nM of 8,9-EET (n = 7) and 1.06 ± 0.37 before and 0.12 ± 0.10 after application of 150 nM of 8,9-EET (n = 5), respectively. In contrast, neither 5-HETE nor 12-HETE or 15-HETE influenced ENaC activity (Fig. 4).

Diclofenac and ibuprofen decrease Na⁺ transport in mpkCCDc14 cells. As demonstrated in Fig. 1, mpkCCDc14 cells produce not only EETs and HETEs, but also significant amounts of prostaglandins. Moreover, PGE₂ and PGF₂α have been reported to increase ENaC Nₚₒ in M-1 cells (49). Fur-
thermore, PGE2 under certain circumstances enhances ENaC activity in A6 (19, 25) and Madin-Darby canine kidney (MDCK) cells (52). Thus we examined whether inhibition of cyclooxygenase (COX) with COX-selective nonsteroid anti-inflammatory agents such as diclofenac and ibuprofen would alter ENaC-mediated sodium transport in mpkCCDc14 cells. Figure 5 summarizes the time course of the inhibition of Na⁺ current in mpkCCDc14 cells in response to treatment with diclofenac and ibuprofen (Fig. 5, A and C, respectively). To determine the fraction of Na⁺ transport mediated by ENaC, 10 μM amiloride was added to the apical surface of the cell monolayer at the end of each experiment. Figure 5, B and D, shows the dose-response relationship for diclofenac- and ibuprofen-induced changes in transepithelial Na⁺ current 4 h after treatment, respectively. The nonsteroid anti-inflammatory agents significantly decreased Na⁺ transport in the mpkCCDc14 cells with IC₅₀ 175 ± 29 and 304 ± 51 μM for diclofenac and ibuprofen, respectively.

To exclude the possibility that the effects of diclofenac and ibuprofen on equivalent short-circuit currents (Iₑ) were mediated by some cytotoxic effect of these drugs, we performed MTT assays on mpkCCDc14 cells. MTT assay measures the conversion of MTT into the purple-colored MTT formazan by the redox activity of living cells, and a decrease in cellular MTT formazan is an index of cell toxicity. The results of these experiments indicated that a high concentration (500 μM) of diclofenac or 1 mM ibuprofen had no effect on cell vitality (Fig. 6). In addition, transepithelial resistance (another indicator of cellular viability) was monitored throughout the entire duration of all electrophysiological measurements of Na⁺ flux in mpkCCDc14 cells. We found that ibuprofen and diclofenac in these concentrations had no effect on transepithelial resistance.

MS-PPOH activates ENaC-mediated sodium transport when cells were pretreated with AA and diclofenac. To demonstrate further that EETs formed by mpkCCDc14 cells are involved in regulation of ENaC-mediated transport, we studied the effects of 11,12-EET and the selective inhibitor of the epoxygenase activity, MS-PPOH, on equivalent Iₑ. Initial experiments tested whether 11,12-EET and MS-PPOH modulate basal ENaC-mediated sodium transport through mpkCCDc14 monolayers. Interestingly, neither 11,12-EET nor MS-PPOH altered equivalent Iₑ in mpkCCDc14 cells under basal conditions (Figs. 7, A and B, respectively). Thus we have designed a protocol whereby MS-PPOH was applied when the cells where stimulated to produce eicosanoids with exogenous AA in the presence of diclofenac, an inhibitor of COX. Application of MS-PPOH (15 μM) in the presence of AA (10 μM) and diclofenac (200 μM) significantly enhanced equivalent Iₑ (Fig. 8). AA given alone increased Iₑ, and this effect was blocked by diclofenac (200 μM), which inhibits COX activity. Similarly, application of AA rapidly increased Iₑ in MDCK-C7 cells, and this effect was blocked by preincubation with the COX inhibitor indo- methacin (30). Thus, under conditions in which eicosanoid production is stimulated by the availability of exogenous substrate, subsequent administration of MS-PPOH increased sodium transport compared with the cells treated with AA and diclofenac alone.

Transient transfection with Cyp2c8 but not Cyp4a10 significantly decreases activity of ENaC overexpressed in CHO cells via cAMP-dependent protein kinase (PKA). The production of EETs and 20-HETE is catalyzed by members of the cytochrome P-450 2C and 4A families, respectively. Patch-clamp experiments were employed to determine the influence of...
overexpression of these isoforms on ENaC activity in CHO cells transiently transfected with α-, β-, and γ-subunits of ENaC. Currents were elicited by voltage ramping from 60 mV down to −100 mV (holding potential 40 mV) as described previously (31, 40). Overexpression of the Cyp4a10 isoform did not affect the amiloride-sensitive current (Fig. 9A). In contrast, upregulation of Cyp2c8 expression significantly decreased ENaC activity (Fig. 9A). Cyp2c8 produces the 14,15- and 11,12-EETs (7). LC/MS analysis identified that the level of 14,15-EET increased in CHO cells that overexpressed Cyp2c8 and 11,12-EETs (7). LC/MS-PPOH blocked the decrease in ENaC activity seen in CHO cells transfected with Cyp2C8. As seen in Fig. 9B, MS-PPOH blocked the decrease in ENaC activity seen in these cells. These results suggest that endogenously formed EETs generated by enzymes of the CYP2C family can modulate the activity of ENaC channels and Na⁺ transport in the CCD.

Additional studies were performed to examine the effects of PKI (14–22) amide, a cell-permeable highly specific inhibitor of cAMP-dependent protein kinase (PKA) on Cyp2c8 modulation of channel activity. As summarized in Fig. 9C, PKI (2 μM) attenuated the Cyp2c8-mediated decrease in ENaC activity. Moreover, pretreatment of ENaC with 11,12-EET for 4 h mimicked the effect of Cyp2c8 and significantly decreased ENaC activity in CHO cells (Fig. 9D).

EETs biphasically decrease ENaC activity initially by affecting channel Po and later by modifying the number of active channels in the membrane. It is generally accepted that there are three primary ways to affect channel’s activity: 1) by altering channel gating (Po); 2) by altering the expression levels of the protein in the membrane (N); and 3) by affecting the conductance of the channel. As seen in Figs. 2 and 3, EETs have no effect on the conductance of the channel. To discriminate between the effect on Po and N, we used single-channel analysis and a biotinylation approach to explore the effects of EETs on the expression of ENaC in the plasma membrane. Figure 10A demonstrates current traces recorded in cell-attached patches from CHO cells expressing ENaC alone (top trace) or coexpressed with Cyp2c8 (bottom trace) at a holding potential of −60 mV. The mean number of active channels within patches was significantly decreased when the channel’s subunits were coexpressed with Cyp2c8 (Fig. 10B), whereas Po remained unchanged. Figure 10C shows a representative Western blot probed with an anti-Myc antibody containing whole cell lysate of CHO cells overexpressing Myc-tagged α-, β-, and γ-ENaC subunits alone or in cells transfected with Cyp2c8 or treated with 11,12-EET (150 nM) for 4 h and 10 min, respectively. As seen in Fig. 10, acute application of EETs had no effect on ENaC protein levels in the membrane, whereas long-term treatment with EETs or transfection of the cells with Cyp2C8 to increase the formation of EETs in the cells significantly decreased the expression of ENaC protein in the plasma membrane.

![Figure 8](http://ajprenal.physiology.org/)

**Figure 8.** Inhibition of cytochrome P-450 (CYP450) epoxygenase with MS-PPOH-activated ENaC-mediated sodium transport when cells were pretreated with AA and diclofenac. Time course of changes in relative Na⁺ transport is shown in response to AA or AA plus diclofenac either alone or together with MS-PPOH. For these experiments AA (green) and vehicle (control; black) were added at time 0. After 30-min and 1-h point measurements, diclofenac and MS-PPOH were subsequently added. Current was normalized to the starting level, and amiloride (10 μM, arrow) was added to the apical membrane at the end of the experiment. The numbers of experiments were at least 6 for each measurement. *P < 0.05 red vs. blue at 4 h.

![Figure 9](http://ajprenal.physiology.org/)

**Figure 9.** Effect of Cyp4a10 and Cyp2c8 on ENaC activity. Summary graph is shown of the means ± SE amiloride-sensitive current density at −80 mV for voltage-clamped Chinese hamster ovary (CHO) cells expressing ENaC subunits in the absence and presence of Cyp4a10 (A) or Cyp2c8 (B and C) not treated and pretreated with CYP450 epoxygenase inhibitor MS-PPOH (15 μM, 4 h; B) or PKA inhibitor (PKI, 2 μM, 1 h; C). D: summary graph of the amiloride-sensitive current for ENaC not treated and pretreated for 4 h with 11,12-EET. The numbers of observations for each group are shown. *P < 0.05 vs. ENaC alone or ENaC + Cyp2c8.
activity. However, the results of the present study indicate that 8,9-EET and 14,15-EET are equipotent as 11,12-EET in inhibiting ENaC activity in mpkCCDc14 cells in our hands. In contrast to the present study, Wei et al. (53) have shown that addition of 5,6-, 8,9-, or 14,15-EET failed to decrease ENaC activity in freshly isolated rat CCD cells. We propose that this may reflect species differences in the regulation of ENaC activity in the CCD of mice vs. rats or differences in the rate of metabolism of the regioisomers of EETs in the intact CCD of rat vs. cultured mice CCD cells. For example, previous studies have indicated that the basal production and levels of eicosanoids differ in various tissues and the endogenous production has to be blocked before the effects of exogenous administration of the various compounds could be demonstrated. This is not to say that the fundamental mechanisms involved in the regulation of ENaC activity are different in these two models but rather that the dynamic equilibrium between EETs involved in ENaC regulation may differ in these murine cells. Consistent with the previous results, we failed to find any effect of 5-, 12/8-, or 15-HETE on ENaC activity. This indicates that EETs but not HETEs regulate Na\(^+\) transport and ENaC activity in the CCD.

Interestingly, our data demonstrate some discrepancies between the effects of 11,12-EET on single channels and absence of effects of MS-PPOH and 11,12-EET on equivalent \(I_c\). As we described in RESULTS, under conditions in which eicosanoid production is stimulated by the availability of an exogenous substrate, subsequent administration of MS-PPOH increased sodium transport compared with the cells treated with AA and diclofenac alone (Fig. 8). The level of endogenous production of AA metabolites is rapidly adjusted in response to exogenous application of eicosanoids. Single-channel recordings allowed us to see rapid changes in activity of one or two channels. In contrast, \(I_c\) represents the effect of not only ENaC but also a variety of transporters. In addition, our first time point recorded is 30 min, and the level of AA metabolites could be already adjusted.

In further experiments, we provide the first evidence that endogenously formed EETs also affect ENaC activity. Moreover, we found that the inhibitory effect of exogenously administered EETs was due to the effect on the gating of the ENaC. However, long-term exposure to EETs or overexpression of ENaC with members of CYP2C-family enzymes also decreases the number of channels expressed in the membrane. These findings are consistent with the results of Carratino et al. (6), who reported that channels with a truncated \(\alpha\), \(\beta\), or \(\gamma\) C terminus were not inhibited by AA or its nonmetabolized analog ETYA and proposed that AA-mediated changes in ENaC functional expression occurred, in part, through changes in the surface expression of ENaC.

In addition to CYP-epoxygenase and CYP-\(\omega\)-hydroxylase, COX has been shown to be able to metabolize AA in the renal tubules, including CCD (34). Recently, it was shown that the COX-dependent AA metabolites such as prostaglandin E (PGE\(_2\)) and prostaglandin F (PGF\(_{2\alpha}\)) activate ENaC in cultured mouse M1 principal cells (49). Moreover, it was shown that infusion of prostaglandins in renal tubular fluid can increase Na\(^+\) reabsorption (3). Addition of exogenous PGE\(_2\) to isosmotic solutions led to large increases in the amiloride-sensitive \(I_c\) and transepithelial conductance in A6 cells (25). PGE\(_2\) also stimulates sodium reabsorption in MDCK-C7 cells (52). Thus
several studies demonstrated that prostaglandins in contrast to EETs stimulate ENaC. However, this is not consistent with the known effects of PGE2 to inhibit Na+ transport in the CCD. For example, Els and Helman (8) reported that PGE2 decreases the NP of Na+ channels by unknown mechanisms. Similarly, it was shown that PGE2 has a biphasic effect, which includes inhibition by acute PGE2 and stimulation by chronic PGE2 exposure in A6 distal nephron cells (19). Acute inhibition of sodium transport by PGE2 was also observed in rabbit cortical collecting tubules (13, 21, 43).

Our data are consistent with the positive influence of prostaglandins on ENaC activity and ENaC-mediated sodium reabsorption. Furthermore, similar to data in microperfused rabbit CCD (48), we did not observe any effect of MS-PPOH on basal amiloride-sensitive sodium transport in mpkCCD14 cells perhaps due to stimulation of transport by prostaglandins. However, application of MS-PPOH in the presence of AA and diclofenac, an inhibitor of COX, significantly enhanced ENaC-mediated sodium reabsorption. Thus we hypothesize that metabolites of AA formed in the CCD have a dual effect on ENaC. Prostaglandins enhance ENaC activity and EETs decrease it. Under different conditions, production of AA metabolites might either stimulate or inhibit sodium transport in the aldosterone-sensitive distal nephron depending on the relative production of EETs vs. prostaglandins.

Previous studies have indicated that 11,12-EET analogs increase cAMP and PKA upregulates renal vascular smooth muscle cell large-conductance calcium-activated K+ channel activity (14). Thus the authors proposed that afferent arteriolar dilation in response to 11,12-EET involves increases in cAMP, PKA activation, and opening of K+ channels. Similarly, elevated EET levels regulate Ca2+ influx into endothelial cells and the subsequent activation of large-conductance calcium-activated K+ channels. This effect occurs via a cAMP/PKA-dependent mechanism that involves the intracellular translocation of TRPC6 channels (9). 11,12-EET is also a potent activator of the vascular KATP channels, and its effects are dependent on the activity of PKA (57). Furthermore, a role for PKA in regulation of ENaC has been reported previously. Activation of PKA has been shown to phosphorylate Nedd4–2 to inhibit ENaC internalization (37). Thus PKA may promote Na+ transport by increasing ENaC expression at the cell surface. Moreover, it was shown that PKA modulates the activity of ENaC by phosphorylation of the β- and γ-subunits (35). Additional mechanisms of ENaC regulation by cAMP/PKA are also proposed. It was previously demonstrated that cAMP/PKA is involved in ENaC exocytosis and modulation of ENaC gating (4, 24, 27, 36, 44, 45). Interestingly, our data revealed that PKA is involved in the negative regulation of ENaC by EETs. It is possible that additional second messengers are recruited in this signaling pathway. For example, it was shown that CFTR regulates ENaC at the level of single-channel gating, by switching the response of single-channel P0 to cAMP/PKA from an increase to a decrease (45).

Thus the present study helps to clarify the complex mechanisms of ENaC regulation by metabolites of AA. We provide evidence that 8,9-, 11,12-, and 14,15-EETs mediate the effects of AA on ENaC. Moreover, EETs and prostaglandins have opposing actions on the ENaC channel. The net effect on Na+ transport and ENaC activity is determined by the balance between the formation of these lipid mediators.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES

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