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Epoxyeicosatrienoic Acids Affect Electrolyte Transport in Renal Tubular Epithelial Cells: Dependence on Cyclooxygenase and Cell Polarity

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Abstract

We investigated the effects of epoxyeicosatrienoic acids (EETs) on ion transport in the polarized renal distal tubular cell line, MDCK C7. Of the four EET regioisomers (5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET) studied, only apical, but not basolateral, application of 5,6-EET increased short circuit current (Isc) with kinetics similar to those of arachidonic acid. The ion transport was blocked by preincubation with the cyclooxygenase inhibitor indomethacin or with the chloride channel blocker NPPB. Further, both a Cl⁻-free bath solution and the Ca²⁺ antagonist verapamil blocked 5,6-EET-induced ion transport. Although the presence of the PGE₂ receptors EP2, EP3, and EP4 was demonstrated, apically added PGE₂ was ineffective and basolaterally added PGE₂ caused a different kinetics in ion transport compared to 5,6-EET. Moreover, PGE₂ synthesis in MDCK C7 cells was unaffected by 5,6-EET treatment. GC/MS/MS analysis of cell supernatants revealed the presence of the biologically inactive 5,6-dihydroxy-PGE₁ in 5,6-EET-treated cells, but not in control cells. Indomethacin suppressed the formation of 5,6-dihydroxy-PGE₁. 5,6-epoxy-PGE₁ the precursor of 5,6-dihydroxy-PGE₁, caused a similar ion transport as 5,6-EET. Cytochrome P450 enzymes homolog to human CYP2C8, CYP2C9, and CYP2J2 protein were detected immunologically in the MDCK C7 cells. Our findings suggest that 5,6-EET affects Cl-transport in renal distal tubular cells independent of PGE₂ but by a mechanism, dependent on its conversion to 5,6-epoxy-PGE₁ by cyclooxygenase. We suggest a role for this P450 epoxygenase product in the regulation of electrolyte transport, especially as a saluretic compound acting from the luminal side of tubular cells in the mammalian kidney.

Keywords

kidney; CYP450; prostaglandin; collecting duct

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INTRODUCTION

In the kidney, arachidonic acid (AA) regulates Cl^- transport in rabbit proximal tubule (23), and rat cortical collecting duct (41). In addition to direct effects of AA, metabolism of AA to bioactive lipid mediators is known to occur by different enzymatic pathways. The cyclooxygenase (COX) and lipoxygenase pathways lead to formation of prostaglandins, prostacyclin, thromboxane and leukotrienes, well known modulators of renal vascular perfusion and electrogenic ion transport (for review see (2)). The cytochrome P450 (P450) monooxygenase pathway including enzymes of the CYP1A, CYP2B, CYP2C, CYP2E, and CYP2J subfamilies (34) catalyze the formation of 4 regioisomeric products, 5,6-, 8,9-, 11,12-, and 14,15-epoxyeicosatrienoic acid (EET) and several of these epoxygenases are expressed in rat (19,45), mouse (21), and human (38) kidney. EETs have a relatively short half-life in vivo and in addition to being incorporated into membrane phospholipids (40) they are rapidly metabolized by the soluble epoxide hydrolase (sEH) to dihydroxyeicosatrienoic acids (DHETs) (46) and/or by β -oxidation to short chain epoxides (10). 5,6-EET is the least chemically stable and is hydrolyzed to 5,6-DHET and its δ -lactone under neutral and acidic conditions; the other EETs are chemically stable in alkaline aqueous solutions (11). EETs have attributed a number of biological activities including vasodilation (31), modulation of renovascular resistance (2), and release of catecholamines (17). EETs are discussed as candidate molecules for the endothelium-derived hyperpolarizing factor (4). However, EETs also exert numerous membrane potential-independent effects on endothelial cell signaling. Recently, a role of 5,6-EET and 8,9-EET as angiogenic lipids has been demonstrated (27). Among the biological activities attributed to EETs, those of significance to the renal regulation of salt and water include inhibition of Na^+ reabsorption in the proximal tubule and collecting duct (20,35), K^+ secretion in the cortical collecting tubule (20), and inhibition of vasopressin-stimulated water transport (18). EETs derived from the kidney were found in human urine (8) and urinary excretion of EET metabolites is increased in pregnancy-induced hypertension (8). Moreover, renal epoxygenase activity is strongly enhanced in response to increased NaCl intake (32) and deficient production of 5,6-EET by epoxygenase inhibition has been associated with salt-sensitive hypertension (24). The CYP2J enzymes, which contribute to EET generation in the kidney, are abundantly expressed within tubules of the mouse renal cortex and outer medulla (22). Importantly, increased CYP2J expression and EET formation has been reported in the spontaneously hypertensive rat kidney (45), data taken to imply that EETs play an important role in regulating renal tubular ion transport.

In this study, we characterized the effects of EETs on ion transport in a hormone-sensitive model of collecting duct principal cells using polarized Madin Darby canine kidney (MDCK) C7 cells (12). In electrophysiological Ussing experiments we found that 5,6-EET to activate a transient electrogenic Cl^- transport. We provide evidence that this ion transport is exclusively activated from the apical (luminal) membrane of the epithelial cell and is dependent on conversion of 5,6-EET by COX to an active metabolite, most likely 5,6-epoxy-PGE₁.

MATERIALS AND METHODS

Cell culture

Madin Darby canine kidney (MDCK) cells of the C7 subtype between passages 74 to 80 were used. They were kindly provided by Dr. Michael Gekle (Physiologisches Institut, Universität Würzburg, Germany) and have properties of distal tubular principal cells (12). The cells were passaged twice a week after reaching confluence and were grown under standard cell culture conditions (37°C and 5% CO_2 atmosphere) in Earle's MEM medium (PAA Laboratories, Cölbe, Germany) supplemented with L-glutamine, 10% fetal calf serum

(Greiner, Frickenhausen, Germany), 100 U/ml penicillin, 100 µg/ml streptomycin, and 1 mM sodium bicarbonate (PAA). For electrophysiological experiments, 5×10^5 cells were seeded in 12 mm culture plate well inserts (Millicell™-HA, Millipore, Eschborn, Germany) and medium was replaced twice a week. Under these conditions MDCK C7 cells form a polarized, high-resistance epithelial monolayer on the Millicell™ microporous membrane within 5–7 days. For cell culture experiments, cells were seeded in 6-well plates and cultured until confluent. Cells were washed and experiments were performed in PBS. Inhibitors were added to the cell culture 30 minutes before addition of EETs.

Ussing experiments

Millicell™ inserts were mounted in specially designed Ussing-type chambers (Feinmechanische Werkstatt, Universität Marburg) exposing 0.6 cm^2 surface area. The cells were bathed on both sides with 8 ml of bath solution (37°C) circulated by a bubble-lift of carbogen (95% O_2 , 5% CO_2) and maintained at pH 7.4. Short circuit current (I_{sc}), transepithelial potential difference (PD), and tissue resistance (RTE) measurements were performed as previously described (42) using a computer-controlled CVC6 voltage clamp device (Fa. Fiebig, Berlin, Germany). All measurements were corrected for bath resistance. Agar-saline bridges and Ag/AgCl-electrodes (Argenthal® Elektrolyt 9823, Fa. Ingold, Germany) served as electrical connections between the Ussing chamber and the voltage clamp device. Tissue equilibration time of 20 minutes was sufficient to obtain stable baseline currents and only C7 cells exhibiting a transepithelial resistance of more than $1000 \Omega \cdot \text{cm}^2$ were used throughout the experiments.

Solutions and drugs

All experiments were carried out in a bath solution containing Na^+ 140.0 mmol/l, Cl^- 123.8 mmol/l, K^+ 5.4 mmol/l, HPO_4^{2-} 2.4 mmol/l, Ca^{2+} 1.2 mmol/l, H_2PO_4^- 0.6 mmol/l, Mg^{2+} 1.2 mmol/l, HCO_3^- 21.0 mmol/l, and glucose 10 mmol/l. For Cl^- -free experiments, Cl^- was substituted with glucuronic acid in equimolar concentrations. Arachidonic acid (in ethanol) and EETs (in acetonitrile) were obtained from Cayman Chemical (Ann Arbor, USA); all other drugs came from Sigma Chemicals (Deisenhofen, Germany) and were dissolved in bath solution.

Sample preparation for LC/MS

For determination of the 4 EETs and the corresponding DHETs by LC/MS analysis, samples were prepared as described recently (25).

Sample preparation for GC/MS/MS

For determination of 5,6-EET metabolites, MDCK C7 cells were grown in 145 mm tissue culture dishes (Greiner, Frickenhausen, Germany). After reaching confluence, 5,6-EET (0.3 µM in acetonitrile) or acetonitrile (control) were added to the medium. Following an additional 2 minutes (the time when maximal electrophysiological effects were observed) the reaction was stopped by adding a 1:1 mixture of ice-cold PBS and ethyl acetate. Likewise, cells were grown in 60 mm dishes to evaluate possible effects of 5,6-EET on intracellular and extracellular PGE_2 levels. Medium (extracellular PGE_2) was collected before (control), 2 and 5 minutes after addition of 1 µM 5,6-EET to the medium. The reaction was stopped by adding a 1:1 mixture of ice cold PBS and ethyl acetate. The cells were collected, briefly vortexed and prepared for gas chromatography - triple stage quadrupole mass spectrometry (GC/MS/MS) determination of 5,6-EET metabolites and intracellular PGE_2 . Briefly, the samples were acidified with formic acid (10%) to pH 3.2 and O-methylhydroxylamine hydrochloride (130 mg) in 1.5 ml acetate buffer (1.5 M, pH 5) was added to form the methoxime. After acidification with formic acid to pH 2.5, the prostanoid

derivatives were extracted twice with 3 ml ethyl acetate-hexane (70:30, v/v). After evaporation of the solvent, acetonitrile (80 µl), pentafluorobenzyl bromide (7 µl) and N,N-diisopropylethylamine (25 µl) were added. The mixture was allowed to react at 40°C for 25 min. The dry sample was purified by TLC (developing solvent: ethyl acetate-hexane 90:10, v/v). A broad zone (RF 0.03–0.39) was eluted with the TLC developing solvent (800 µl) and water (50 µl) was added. After centrifugation, the ethyl acetate phase was withdrawn, the solvent was evaporated and the prostanoids derivatized with BSTFA (25 µl; 40°C, 1 h). A 2 µl aliquot of this solution was injected.

GC/MS/MS analysis

A Finnigan MAT TSQ700 tandem mass spectrometer equipped with a Varian 3400 gas chromatograph and a CTC A200S autosampler was employed. Gas chromatography of prostanoid derivatives was carried out on a J&W DB-1 (15 m, 0.25 mm i.d., 0.25 µm film thickness) capillary column (Carlo Erba, Hofheim, Germany) in the splitless mode at an inlet pressure of 100 kPa. The oven temperature program for all prostanoids analyzed was as follows: initial temperature of 100°C was held for 2 min, then increased at 30°C per min to 280°C and at 5°C per minutes to 310°C. This temperature was held for 2 min. Mass spectrometer conditions were as follows: interface temperature 300°C, source temperature 150°C, methane CI gas pressure 50 Pa, electron energy 70 eV, emission current 0.4 mA, conversion dynode 20 kV, and electron multiplier 1800 V. Collision-induced decomposition spectra of the [M-PFB][−]-ion of the 5,6- dihydroxy-PGE₁ derivative (m/z 702) were taken at a collision cell pressure of 0.2 Pa and a collision energy of 15 eV.

Preparation of 5,6-epoxy-PGE₁

The 5,6-epoxy-PGE₁ was obtained exactly as described by Sakairi et al. (37) using PGE₂ methyl ester (Cayman Chemicals, Ann Arbor, USA) dissolved in anhydrous CH₂Cl₂ and to which in situ prepared dimethyldioxirane (28) was added. Structure was confirmed by GC/MS/MS.

Preparation of canine kidney sections

We used canine kidney tissue to look for expression and localization of an EET-forming epoxygenase. We focused on CYP2J protein which has already been reported to be expressed in mouse, rat, and human kidney (22). The fresh kidney of a male Rottweiler was obtained after euthanization because of fatal hip dysplasia. Normal renal tissue was snap-frozen in liquid nitrogen and stored at −80°C. For cryosectioning, specimens were embedded in tissue adhesive medium (Tissue-Tek, Sakura Finetek, Torrance, CA). Slices of 5 µm thickness were cut using a Leica CM1900 cryostat and were thaw mounted onto poly- L-lysine-coated slides.

Immunohistochemistry

For CYP2J immunostaining, the avidin:biotinylated enzyme complex (ABC) method was used following the manufacturers protocol (VECTASTAIN® Elite ABC-Peroxidase Kit, Vector Laboratories, Burlingame, CA). Slides were fixed in acetone at 4°C for 10 minutes and then air-dried at room temperature. For quenching of endogenous peroxidase, slides were incubated in 0.3% H₂O₂ in methanol for 30 minutes. Overnight incubation using a polyclonal anti-human CYP2J2 antibody (1:200 dilution in PBS/10% goat serum) was followed by successive incubation at 37°C with a biotinylated secondary antibody, VECTASTAIN® Elite ABC Reagent, and peroxidase substrate solution. Control experiments without primary antibody showed no specific immunostaining. The CYP2J2 antibody was prepared by immunizing rabbits with a partially purified preparation of recombinant CYP2J2 as previously described (44). The antibody has been previously shown

to immunoreact with CYP2J subfamily P450s in rabbit, mouse, and rat, but does not cross-react with non-CYP2J subfamily P450s including members of the CYP1A, CYP2A, CYP2B, CYP2C, CYP2D, CYP2E, and CYP4A subfamilies (22,29,44).

Immunoblotting

MDCK C7 cell pellets and human and rat kidney tissue specimens were disrupted and homogenized using a Polytron homogenizer (Kinematica, Lucerne, Switzerland) and a Potter-Elvehjem homogenizer (Kobe, Marburg, Germany). Total protein was extracted and protein concentrations were determined by the bicinchoninic acid method according to the manufacturer's protocol (Pierce, Rockford, Illinois) using bovine serum albumin as protein standard. Samples of 100 µg protein were separated by SDS-PAGE on 8%–16% polyacrylamide minigels (Gradigel, Frenchs Forest, Australia) and transferred to nitrocellulose membranes (Hybond C extra, Amersham, Little Chalfont, UK). The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline (TBS) and incubated with 5% goat serum (in TBS) for 30 minutes. After washing with 0.1% Tween 20/TBS (TTBS), the primary antibodies (anti-human EP1 to EP4 receptor from Cayman Chemicals, Ann Arbor, USA, anti-human CYP2C8 (26), anti-human CYP2C9 (25), anti-human sEH and anti-human CYP2J2 antibody) were applied for 2 hours, followed by incubation with donkey anti-rabbit IgG peroxidase-conjugate (1:10000). After several washing steps, sites of antibody-antigen reaction were visualized by enhanced chemiluminescence technique (ECL plus, Amersham) with exposure times of 5 seconds to 2 minutes on autoradiographic film (Hyperfilm ECL, Amersham). In control experiments without primary antibody, no protein bands were detected after the staining procedure.

PCR analysis of EP receptor mRNA

mRNA expression of the EP1 to EP4 receptor in cultured MDCK C7 cells was examined by PCR as described (3).

Statistics

Data are presented as mean values $\pm$ S.E. Student's t-test (paired or unpaired, as appropriate) was used to compare data between two groups. To compare data between various groups statistical analysis were performed by one-way ANOVA followed by Bonferroni's multiple comparison test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Effects of AA on MDCK monolayers

MDCK C7 cells seeded in Millicell™ HA culture plate well inserts form a tight and polarized epithelial monolayer within several days. In a first approach we studied the effect of AA on ion transport. In Ussing chamber experiments (Table 1), basolateral AA (1 µM) had no significant effect on short circuit current, potential difference (PD), or transepithelial resistance (R_{TE}). In contrast, addition of AA to the apical bath solution rapidly increased basal I_{sc} . The corresponding PD decreased while R_{TE} remained unchanged. As depicted in an original trace recording (Fig. 1), the maximum effect occurred within 3 minutes and returned to baseline values within 10 minutes of adding AA. Preincubation of MDCK cells with P450 epoxigenase inhibitors ketoconazole (10 µM), clotrimazole (10 µM), or SKF525A (100 µM) for 30 minutes markedly attenuated the effect of AA (Table 1). The electrogenic ion transport was completely blocked by 10 µM indomethacin (Table 1), suggesting that a COX-dependent AA metabolite mediated the observed effect.

Effect of PGE₂ on MDCK monolayers and expression of PGE₂ receptors

Prostaglandin E₂ (PGE₂) is the major COX product of renal arachidonic acid metabolism (2), therefore we studied the effect of PGE₂ under our experimental setting. However, compared to 5,6-EET we observed completely different kinetics for PGE₂ induced ion transport in the Ussing experiments (Fig. 2). Addition of 1 μ M PGE₂ resulted in a biphasic time course with a peak at approximately 2 minutes leading to a stable, amiloride-sensitive ion transport after 5–10 minutes. The addition of the Cl[−] channel blocker 5-nitro-2-(3-phenylpropyl-amino)benzoic acid (NPPB) had no significant effect. The expression of the PGE₂ receptors EP1, EP2, EP3, and EP4 was studied by PCR and western blot analysis. We observed that MDCK C7 cells express for the EP2, EP3, and EP4 receptor mRNA and protein but not for the EP1 receptor (Fig. 3). As reported previously, the effect of PGE₂ can be mimicked by application of the EP2 agonist butaprost (43) suggesting involvement of the EP2 receptor in the PGE₂-dependent ion transport.

Effects of EET regioisomers on MDCK monolayers

In a second approach we studied the effect of EETs on ion transport. Addition of EETs to the bath solution showed selective effects of the different regioisomers: 5,6-, 8,9-, 11,12-, and 14,15-EET. The solvent, acetonitrile, as well as basolateral addition of 5,6-EET up to 3 μ M had no effect on electrophysiological parameters of MDCK C7 monolayers (Table 2). In contrast, apically applied 5,6-EET concentration-dependently increased basal I_{SC}, while PD decreased, and R_{TE} remained unchanged. Significant effects on I_{SC} and PD were observed in a relatively small range beginning at 30 nM (calculated EC₅₀ 168 nM, data not shown). At a concentration of 0.3 μ M almost maximal effects of 5,6-EET were observed. Therefore, this concentration was used in the majority of subsequent experiments. The original trace recording of three experiments showed similar kinetics as were observed using AA (Figure 4A). There was maximal electrogenic transport after 2 to 3 minutes, decreasing to zero within 8 to 10 minutes. The ion transport also was completely dependent on COX, since 10 μ M indomethacin and also 10 μ M diclofenac strongly inhibited the effects of 5,6-EET (Fig. 4B). Furthermore, the EP1 antagonist ONO-8711, and the EP4 antagonist ONO-AE3-208, at concentrations of 10 μ M, had no effect on the 5,6-EET-induced ion transport (Fig. 4B). The more stable 5,6-EET methyl ester was as effective as 5,6-EET, while 5,6-dihydroxyeicosatrienoic acid (5,6-DHET) was biologically inactive (Table 2). Apical addition of 8,9-EET evoked a small but significant increase in I_{SC}, however, this effect was less than 10% of that of the same concentration of 5,6-EET (Table 2). In contrast, 11,12-, and 14,15-EET failed to affect ion transport in MDCK C7 cells at concentrations as high as 3 μ M.

Characterization of ion transport by 5,6-EET

To further characterize the electrogenic ion transport induced by 5,6-EET, several established inhibitors of epithelial Cl[−] and Na⁺ transport were used. Preincubation with furosemide (furo; 0.3 mM), an inhibitor of Na-K-2Cl cotransport, and hydrochlorothiazide (HCT; 0.1 mM), which is known to block the apical Na-Cl cotransporter, had no effect on I_{SC} stimulation by 5,6-EET (Fig. 5). Similar responses were observed using the epithelial sodium channel (ENaC) blocker, amiloride. In contrast, the Cl[−] channel inhibitor NPPB, and use of Cl[−]-free buffer strongly inhibited the increase of I_{SC} by 90% and 82%, respectively, when compared to 5,6-EET alone. Previous studies suggest a crucial role of extracellular Ca²⁺ influx in mediating the effects of 5,6-EET (36) and in support of these findings, preincubation with the L-type voltage-gated Ca²⁺ channel blocker verapamil (100 μ M) significantly inhibited the 5,6-EET effect (Fig. 5).

LC/MS determination of EETs and PGE₂

We studied the formation of EETs and PGE₂ by MDCK C7 cells from exogenously added arachidonic acid by MDCK C7 cells by means of LC/MS analysis (Fig. 6A). Following incubation with arachidonic acid (100 μ M) 5,6-EET was generated, however 8,9-EET and 11,12-EET were not detectable and only small amounts of 14,15-EET were found. Regarding the dihydroxy metabolites, only 14,15-DHET was detected. Furthermore, arachidonic acid was converted to large amounts of PGE₂ (Fig. 6A).

GC/MS/MS determination of PGE₂ and 5,6-EET metabolites

Several investigations have shown that modulation of ion transport by 5,6-EET is probably mediated by stimulation of endogenous PG biosynthesis (30,37). Therefore, we examined the formation of PGE₂ in MDCK cells after incubation with 5,6-EET (0.3 μ M). GC/MS/MS analysis of pelleted and washed MDCK C7 cells revealed no difference in intracellular PGE₂ levels between to control (untreated) cells and cells incubated with 5,6-EET for 2 and 5 minutes (Fig. 6B). In the extracellular medium, PGE₂ levels were higher, but again no difference between control and 5,6-EET-treated cells was detectable. Other prostanoids, including 6-keto-PGF_{1 α} and TXB₂, were present in much lower amounts, but also did not differ between control and 5,6-EET-treated cells (data not shown).

To further study the metabolism of 5,6-EET in this system, MDCK C7 monolayers were exposed to either solvent or 5,6-EET. In contrast to control cells, MDCK C7 cells treated with apical 5,6-EET for 2 minutes showed a specific peak in the gas chromatogram identified by its product ion spectrum as 5,6-dihydroxy-PGE₁, the stable hydrolysis product of 5,6-epoxy-PGE₁ (Fig. 6C). Following incubation of the EET-treated cells with the COX inhibitor indomethacin the 5,6-dihydroxy-PGE₁ peak disappeared (Fig. 6D). When MDCK cells were stimulated with arachidonic acid in the presence of the P450 inhibitor SKF525A, the 5,6-dihydroxy-PGE₁ peak was also diminished (Fig. 6D). However, electrophysiological experiments with 5,6-dihydroxy-PGE₁, revealed that it exhibits no electrogenic activity (Tab.3). Therefore, 5,6-epoxy-PGE₁, the unstable precursor of 5,6-dihydroxy-PGE₁ was synthesized and analyzed by Ussing technique. Following apical addition, a significant increase in I_{SC} was observed with a similar pattern of ion transport as 5,6-EET (Tab. 3).

Analysis of CYP2C and CYP2J protein expression

Epoxygenases of the CYP2C subfamily have been shown to oxidize arachidonic acid to various EETs in liver and kidney and CYP2J enzymes have been proposed to be responsible for AA metabolism to EETs in mouse and rat kidney (22,45). Therefore, we examined CYP2C and CYP2J expression in MDCK C7 cells (Fig. 7A&B). Enzymes, with a molecular mass similar to that of the human CYP2C isoforms, CYP2C8 and CYP2C9, were expressed by MDCK cells (Fig. 7A). Similarly, immunoblotting using a specific CYP2J2 antibody revealed the expression of a single protein band with an apparent molecular mass of 56 kD in MDCK C7 cells, human kidney, and rat kidney (Fig 7A). Moreover, by immunocytochemistry we observed strong staining of blood vessels and collecting ducts in the renal cortex (Fig. 7B), while other tubular structures exhibited no significant immunostaining.

Expression and activity of soluble epoxide hydrolase in MDCK cells

The soluble epoxide hydrolase (sEH) is known to metabolize EETs to the corresponding dihydroxy metabolites. To determine whether the SEH affects 5,6-EET metabolism in MDCK C7 cells we examined enzyme expression and activity. Using a specific antibody directed against the human sEH enzyme we observed strong staining of a protein band with similar electrophoretic mobility as a recombinant sEH protein (Fig. 7A). As 14,15,-EET is

known to be a good substrate for sEH we compared the metabolism of 14,15-EET and 5,6-EET. Following application of 14,15-EET to cultured MDCK C7 cells the sEH product 14,15-DHET was detectable in the supernatant (Fig. 8). Moreover, the 14,15-DHET formation was suppressed by more than 90% by preincubation selective inhibitors of sEH, 12-(3-adamantan-1-yl-ureido)dodecanoic acid (AUDA) or 1-cyclohexyl-3-dodecylurea (CDU). In contrast, no significant decrease in the conversion of 5,6-EET to 5,6-DHET was observed following sEH inhibitor treatment. This indicates that 5,6-EET is a minor substrate for sEH as has been previously reported (46).

DISCUSSION

The results of the present investigation show that AA and 5,6-EET elicit electrogenic transport in MDCK C7 cells, effects dependent on COX and Cl^- channel activity. As MDCK C7 cells express the P450 epoxygenases of the CYP2C and CYP2J families we propose that AA is metabolized by one or more of these P450s to 5,6-EET, which is then converted by cellular COX to 5,6-epoxy-PGE₁. The latter unstable EET product acts directly or indirectly on Cl^- channels to increase their activity. 5,6-epoxy-PGE₁ rapidly decomposes to 5,6-dihydroxy-PGE₁ a fact that accounts for the detection of 5,6-dihydroxy-PGE₁ in MDCK C7 cells following application of AA or 5,6-EET. Although the sEH is the major pathway for the inactivation of EETs and is expressed by the MDCK C7 cells, our data do not support a major role for this enzyme in the enzymatic inactivation of 5,6-EET.

Under our experimental conditions, 5,6-EET and its methyl ester, but not 8,9-, 11,12- or 14,15-EET, exerted strong electrogenic effects and only apical application of 5,6-EET was able to stimulate ion transport. We excluded PGE₂ as a mediator of the observed 5,6-EET-stimulated effects for five reasons: First, compared to 5,6-EET, the apical application of PGE₂ induced only a marginal increase in *I*_{sc}. Second, the *I*_{sc} kinetics evoked by PGE₂ was quite different from the kinetics elicited by AA or 5,6-EET. In particular, there was a strict polarity in the actions of these eicosanoids; the apical action of 5,6-EET was in contrast to the basolateral action of PGE₂. Third, the effect of PGE₂ was sensitive to amiloride (10 μM), indicating the involvement of Na^+ fluxes, most likely via Na^+ channels (IC_{50} of amiloride for Na^+ channel is 0.1–0.5 μM , for the Na^+/H^+ antiporter 5–50 μM , and for the $\text{Na}^+/\text{Ca}^{2+}$ exchanger approximately 1100 μM (39)). In contrast the effect of 5,6-EET was insensitive to amiloride. Fourth, 5,6-EET had no effect on the cellular formation of PGE₂; moreover, 5,6-EET also not provoke the formation of other EETs. Fifth, 5,6-epoxy-PGE₁, a cyclooxygenase product of 5,6-EET evoked a similar change in ion transport as AA or 5,6-EET.

Our experiments using different ion transport inhibitors indicate that 5,6-EET increases apical Cl^- secretion in MDCK C7 cells suggesting that this eicosanoid has saluretic properties in the distal tubule. It is not known whether or not 5,6-EET also demonstrates saluretic properties *in vivo*, however, 5,6-EET-induced saluresis would fit well with the proposed antihypertensive role EETs. Excess dietary salt induces a P450 epoxygenase expression in rat kidneys and treatment with the epoxygenase inhibitor, clotrimazole, produces significant increases in mean arterial blood pressure (24). It has been suggested that the salt-inducible renal epoxygenase pathway may be one of the functionally significant components of the adaptive response to increased salt intake. In support of this assumption 5,6-EET has been shown to be a powerful inhibitor of Na^+ absorption in the distal nephron (20). Interestingly, elevated levels of 8,9-DHET and 11,12-DHET, the stable metabolites of 8,9-EET and 11,12-EET, were found in urine samples from patients with pregnancy-induced hypertension (8). Although it is unknown whether increases in EET levels are causative or compensatory with respect to hypertension, it might be possible that different EET isoforms are involved in different aspects of blood pressure regulation.

In the rat kidney, 5,6-EET has been shown to mediate the renal vasodilation to arachidonic acid. This effect was unmasked by inhibition of COX, which abolished the PGH₂-mediated vasoconstrictor effect (33). This indicates that 5,6-EET itself and not a COX product of 5,6-EET mediates the reported vasodilator effect. In our model 5,6-EET itself did not affect Cl⁻ efflux while its COX product, 5,6-epoxy-PGE₁, appears to be the active agent. Importantly, both effects (vasodilation and electrogenic effects) would be expected to lower blood pressure.

Electrogenic ion transport stimulated by 5,6-EET, but not by other EETs, has been demonstrated in isolated-perfused collecting ducts from rabbits (37) and 5,6-EET depolarized transepithelial voltage (V_T) in association with an increase in $[Ca^{2+}]_i$. However, there are three striking differences in that study compared to our findings. First, the authors observed that 5,6-EET stimulated PGE₂ production severalfold in the perfused collecting ducts and therefore indomethacin completely blocked the effect of 5,6-EET on V_T . Second, both addition of 5,6-EET from luminal and basolateral affected $[Ca^{2+}]_i$. Third, the authors observed that PGE₂ caused a similar effect on V_T as 5,6-EET and that 5,6-epoxy-PGE₁ was ineffective under their experimental setting. In our study the electrogenic effect of 5,6-EET in MDCK C7 cells was also sensitive to COX inhibition, however, PGE₂ levels were not altered following 5,6-EET addition. Moreover, PGE₂ affected V_T different to 5,6-EET. Therefore, we conclude that in MDCK C7 cells ion transport evoked by 5,6-EET (or AA) is dependent on formation of another metabolite generated by COX, 5,6-epoxy-PGE₁. Its hydration product 5,6-dihydroxy-PGE₁ was detected in the supernatants of 5,6-EET (or AA) treated cells. Furthermore, only the apical application of 5,6-EET induced Cl⁻ secretion. This is in direct contrast to the observations that exogenous PGE₂ induces a distinct I_{SC} pattern and that only following its basolateral application. This might explain why 5,6-EET in the basolateral bathing solution did not affect Cl⁻ transport in the microperfused medullary thick ascending limb of the rat (15). Interestingly, 5,6-EET also requires its conversion by COX in order to exert its vasoactivity in the rat caudal artery (6). The renal vasodilator effects of 5,6-EET, on the other hand, have been attributed to both the COX-dependent conversion of 5,6-EET to 5,6-epoxy-PGE₁ as well as the stimulation of PGE₂ generation by 5,6-EET (5).

The mechanism by which 5,6-EET increases Cl⁻ secretion is currently unknown. Several investigations point to an increase in $[Ca^{2+}]_i$ as part of the signalling mechanism involved (14,16,23). Accordingly, preventing influx of extracellular Ca²⁺ by the voltage-gated Ca²⁺ channel blocker verapamil strongly inhibited the effects of 5,6-EET in our study. The activation of Ca²⁺-dependent K⁺ channels might also be involved in mediating the actions of 5,6-EET (1,31). In the rat colon, we observed that 5,6-EET indirectly induces Cl⁻ secretion via a mechanism dependent on the activation of basolateral K_{Ca} channels (unpublished observation). Of note, K_{Ca} channels are known to be located in the apical membrane of collecting tubule principal cells (13). Further studies are necessary to identify the precise molecular mechanism by which 5,6-EET affects ion transport in MDCK C7 cells.

CYP2C and 2J generate EETs and are abundantly expressed within tubules of the mouse renal cortex and outer medulla (22) and increased CYP2J expression and EET formation was observed in the spontaneously hypertensive rat kidney (45). Immunohistochemical analysis and western blotting studies demonstrated abundant expression of CYP2J immunoreactive protein in canine kidney blood vessels and in the cortical collecting duct, as well as in the MDCK C7 cell line. In addition we observed expression of two enzymes of the CYP2C subfamily, CYP2C8 and CYP2C9 in MDCK C7 cells. Though other P450s may also contribute to EET biosynthesis, we speculate that CYP2J- and/or CYP2C-derived EETs may play an important role in regulation of renal tubular ion transport. Since renal tissue express both COX and P450 epoxygenases (22), the metabolism of AA to 5,6-EET and

further conversion to 5,6-epoxy-PGE₁ seems likely. We propose that rapid non-enzymatic hydrolysis to the stable inactive 5,6-dihydroxy-PGE₁ (7,37) may represent a “switch-off” mechanism for the 5,6-EET signal.

It remains unknown whether 5,6-epoxy-PGE₁ acts directly on channel activity or indirectly as a second messenger. As an example for the latter, 14,15-EET serves as an intracellular second messenger for EGF involving the activation of kinase-associated mitogenic pathways in the renal LLCPKc14 cells (9). Another possibility would be the binding to specific EET receptors, although the identity of these putative receptors remains unknown. We exclude the activation of PGE₂ receptors as PGE₂ evoked a Na⁺ channel dependent of ion transport with different kinetics compared to 5,6-EET. The strict basolateral action indicate that the relevant PGE₂ receptors are located on the basolateral side of the cells, whereas 5,6-EET acts on the apical membrane. Although we demonstrated the presence of EP2, EP3, and EP4 receptor in MDCK C7 cells, at least EP1 and EP4 antagonists were unable to block the effects of 5,6-EET. Interestingly, PGE₁ does not differ from PGE₂ but does differ from 5,6-EET with regard to stimulation of amiloride-sensitive Na⁺ reabsorption in MDCK C7 cells (data not shown).

In conclusion, apical application 5,6-EET induces Cl⁻ transport in MDCK C7 cells, a model of renal collecting duct principal cells. We provide evidence that 5,6-EET is a substrate for COX forming the Cl⁻ transport-activating metabolite 5,6-epoxy-PGE₁, which is then further metabolized to the corresponding inactive 5,6-dihydroxy-PGE₁. The role of 5,6-EET might have been underestimated due to its rapid *in vivo* decomposition and in light of difficulties in its detection. Since EETs are also found in human urine (8), the possibility of a saluretic compound acting predominantly from the luminal side of tubular cells is intriguing.

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Figure 1.

Original trace recording showing the effect of 1 μM arachidonic acid on short circuit current (I_{sc}) of indomethacin-pretreated (10 μM , chambers 1–3) and control (chambers 4–6) MDCK C7 monolayers. Note time course and COX-sensitivity of ion transport. Cells were seeded on microporous membranes until forming a high-resistance epithelial monolayer. Membranes were mounted in Ussing-type chambers and bathed on both sides. Time point of addition of indomethacin and arachidonic acid is indicated in the figure.

Figure 2.

Original trace recording showing the effect of basolateral PGE₂ (1 μ M) on short circuit current of MDCK C7 monolayers. Note different time course compared to AA (Fig. 1), and 5,6-EET (Fig. 4B), as well as sensitivity of ion transport to epithelial sodium channel blocker, amiloride, but not Cl⁻ channel blocker, NPPB (5-nitro-2-(3-phenylpropyl-amino)benzoic acid). Cells were seeded on microporous membranes until forming a high-resistance epithelial monolayer. Membranes were mounted in Ussing-type chambers and bathed on both sides. Time point of addition of PGE₂, NPPB, and amiloride is indicated in the figure. A representative of four independent experiments is shown.

Figure 3.

Expression of EP receptor mRNA and protein in MDCK C7 cells. mRNA expression (above) was analyzed by PCR using EP receptor specific oligonucleotides and EP receptor protein (below) expression was studied by immunoblotting using specific EP receptor antibodies. The observed apparent molecular weights are 62, 65, and 72 kD for the EP2, EP3, and EP4 receptor, respectively. Note that neither EP1 mRNA nor EP1 protein was detected.

Figure 4A

Figure 4B

Figure 4.

(A) Effect of apical application of 5,6-EET (0.3 μM) on short circuit current (I_{sc}). Cells were seeded on microporous membranes until forming a high-resistance epithelial monolayer. Membranes were mounted in Ussing-type chambers and bathed on both sides. Time point of apical addition of 5,6-EET is indicated in the figure. Representative three stimulations (chamber 1–3) out of $n=6$ are shown. (B) Effect of indomethacin (10 μM), diclofenac (10 μM), the EP1 antagonist ONO-8711 (10 μM) and the EP4 antagonist ONO-AE3-208 (10

μM) on 5,6-EET induced ion transport. The inhibitors and the antagonists, respectively, were added to apical bath solution 20 minutes prior to 5,6-EET addition (n=6). *, p<0.05.

Figure 5.

Characterization of the effect of 0.3 μ M 5,6-EET on short circuit current (I_{sc}) of MDCK C7 cell monolayers using established ion transport inhibitors/blockers. Cells were seeded on microporous membranes until forming a high-resistance epithelial monolayer. Membranes were mounted in Ussing-type chambers and bathed on both sides. Bars indicate apical 5,6-EET alone ($n = 7$) as control (100%), and after preincubation for 10 minutes with 0.3 mM furosemide (furo, $n = 4$), 0.1 mM hydrochlorothiazide (HCT, $n = 4$), 10 μ M amiloride (ami, $n = 4$), 10 μ M 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB, $n = 3$), 100 μ M verapamil (vera, $n = 4$), and use of chloride-free bath solution (Cl-free, $n = 4$). *, $p < 0.05$.

Figure 6A

Figure 6B

m/z	fragment
702	[P] ⁻
612	[M - TMSOH] ⁻
580	[M - TMSOH - CH ₃ OH] ⁻
522	[M - 2 TMSOH] ⁻
492	[M - 2 TMSOH - CH ₂ O] ⁻
450	[M - 2 TMSOH - (CH ₃) ₂ Si=CH ₂] ⁻
432	[M - 3 TMSOH] ⁻
402	[M - 3 TMSOH - CH ₂ O] ⁻
360	[M - 3 TMSOH - (CH ₃) ₂ Si=CH ₂] ⁻
342	[M - 4 TMSOH] ⁻
330	[M - 3 TMSOH - (CH ₃) ₂ Si=CH ₂ - CH ₂ O] ⁻
310	[M - 4 TMSOH - CH ₃ OH] ⁻
298	[M - 4 TMSOH - CO ₂] ⁻
266	[M - 4 TMSOH - CH ₃ OH - CO ₂] ⁻

Figure 6C

Figure 6D

Figure 6.

(A) Formation of the 4 EETs, their hydration products DHETs and of PGE₂ following addition of 100 μM arachidonic acid. MDCK C7 cells were cultured until confluent, washed and arachidonic acid was added with buffer for 30 min. Thereafter supernatants were removed and EETs, DHETs, and PGE₂ were quantified by LC/MS (n = 4). (B) Effect of 5,6-EET (1 μM) on intracellular and extracellular PGE₂ levels of MDCK C7 cells as determined in duplicate by GC/MS/MS. MDCK C7 cells were cultured until confluent. Cells and medium were collected before (0 minutes), and after apical addition of 5,6-EET for 2 and 5 minutes, respectively, according to the maximal effect on ion transport (n = 3 for each group). (C) Product ion spectrum of the GC peak observed in 5,6-EET-treated MDCK C7 cells. The fragments identified this metabolite as 5,6-dihydroxy-PGE₁. TMS, trimethylsilylether; PFB pentafluorobenzylester; parent ion [P]⁻ = [M - PFB]⁻; TMSOH =

(CH₃)₃SiOH. (D) Inhibition of 5,6-dihydroxy-PGE₁ (5,6-dh-PGE₁) formation from AA by SKF525A (SKF, 100 μM) and from 5,6-EET by indomethacin (indo, 10 μM). MDCK C7 cells were cultured until confluent, washed and arachidonic acid (AA) or 5,6-EET were added with buffer for 2 minutes. The supernatants were removed and prepared for MS analysis (n = 3). *, p<0.05.

Figure 7A

canine renal cortex

canine renal medulla

Figure 7B

Figure 7.

(A) Immunoblot of CYP2J2 (above) CYP2C8, CYP2C9 (middle), and sEH (below) immunoreactive proteins in MDCK C7 cells (MDCK). Recombinant protein standards for CYP2C8 (2C8), CYP2C9 (2C9), and sEH, are indicated. For CYP2J2 protein the expression in human and rat kidney homogenates is also shown (above). (B) Localization of CYP2J2 immunoreactive protein in canine kidney sections by immunocytochemistry. Positive staining was seen in blood vessels (thin arrows) and also in cortical collecting ducts (thick arrows). Magnification 100x (A), and 400x (B); GLOM, glomerulus, PT, proximal tubule.

Figure 8.

Effect of the sEH inhibitors AUDA (12-(3-adamantan-1-yl-ureido)dodecanoic acid) and CDU (1-cyclohexyl-3-dodecylurea) on formation of DHETs. Cells were grown until confluent, washed and then preincubated with AUDA or CDU (each at 100 μ M) for 30 minutes in renewed buffer. Thereafter 5,6-EET or 14,15-EET were added to the culture medium and supernatants were removed after 15 minutes of incubation. The amount of 5,6-DHET and 14,15-DHET were determined in the supernatants by LC/MS/MS (n= 5). Without EET addition no DHETs were detected (data not shown). *, $p < 0.05$.

Table 1

Effect of arachidonic acid on MDCK C7 monolayers.

agonist	n	basal PD (mV)	experiment	basal I_{sc} ($\mu A/cm^2$)	experiment	basal R_{TE} ($\Omega \cdot cm^2$)	experiment
AA apical	5	0.21 $\pm$ 0.2	-4.1 $\pm$ 0.5 *	-0.17 $\pm$ 0.2	3.4 $\pm$ 0.4 *	1218 $\pm$ 3	1191 $\pm$ 18
AA basolateral	4	-0.36 $\pm$ 0.2	-0.14 $\pm$ 0.3	0.28 $\pm$ 0.1	0.12 $\pm$ 0.2	1255 $\pm$ 25	1256 $\pm$ 25
AA + indomethacin	3	0.03 $\pm$ 0.2	0.08 $\pm$ 0.2 †	-0.04 $\pm$ 0.2	-0.08 $\pm$ 0.2 †	1164 $\pm$ 51	1164 $\pm$ 49
AA + ketoconazole	4	-0.21 $\pm$ 0.1	-1.69 $\pm$ 0.2 *	0.16 $\pm$ 0.1	1.36 $\pm$ 0.2 *	1256 $\pm$ 27	1242 $\pm$ 15
AA + clotrimazole	4	-0.11 $\pm$ 0.2	-1.41 $\pm$ 0.3 *	0.06 $\pm$ 0.2	1.14 $\pm$ 0.3 *	1156 $\pm$ 41	1172 $\pm$ 25
AA + SKF525A	4	0.01 $\pm$ 0.1	-0.69 $\pm$ 0.2 *	0.11 $\pm$ 0.1	0.42 $\pm$ 0.2 *	1226 $\pm$ 37	1236 $\pm$ 19

Effect of free arachidonic acid (AA) on potential difference (PD), short circuit current (I_{sc}) and transepithelial resistance (RTE) of MDCK C7 cells mounted in Ussing-type chambers (mean $\pm$ SE). The final concentration in the bath solution was 1 μM (*, $p < 0.05$ vs. basal; †, $p < 0.05$ vs. apical AA alone). Pretreatment with the COX inhibitor indomethacin (10 μM), and the P450 inhibitors ketoconazole (10 μM), clotrimazole (10 μM), and SKF525A (100 μM) strongly attenuated the effect of apical AA.

Table 2

Effect of EETs on MDCK C7 monolayers.

agonist	n	basal		experiment		basal		experiment		basal		experiment	
		PD (mV)		I _{sc} (μA/cm ²)		I _{sc} (μA/cm ²)		I _{sc} (μA/cm ²)		R _{TE} (Ω·cm ²)		R _{TE} (Ω·cm ²)	
5,6-EET basolateral	6	-0.41 ± 0.2	-0.44 ± 0.1	0.33 ± 0.2	0.36 ± 0.1	0.33 ± 0.2	0.36 ± 0.1	1221 ± 42	1227 ± 36	1221 ± 42	1227 ± 36	1221 ± 42	1227 ± 36
5,6-EET apical	7	-0.32 ± 0.2	-4.02 ± 0.3*	0.26 ± 0.1	3.29 ± 0.2*	0.26 ± 0.1	3.29 ± 0.2*	1240 ± 13	1220 ± 20	1240 ± 13	1220 ± 20	1240 ± 13	1220 ± 20
5,6-EET-Me	4	-1.34 ± 0.2	-4.65 ± 0.5*	1.05 ± 0.1	3.74 ± 0.3*	1.05 ± 0.1	3.74 ± 0.3*	1278 ± 21	1241 ± 23	1278 ± 21	1241 ± 23	1278 ± 21	1241 ± 23
5,6-DHET	3	-1.8 ± 0.1	-2.2 ± 0.4 [†]	1.48 ± 0.1	1.77 ± 0.3 [†]	1.48 ± 0.1	1.77 ± 0.3 [†]	1215 ± 37	1216 ± 35	1215 ± 37	1216 ± 35	1215 ± 37	1216 ± 35
8,9-EET	4	-1.05 ± 0.1	-1.35 ± 0.1*	0.86 ± 0.1	1.08 ± 0.1*	0.86 ± 0.1	1.08 ± 0.1*	1239 ± 29	1240 ± 29	1239 ± 29	1240 ± 29	1239 ± 29	1240 ± 29
11,12-EET	4	-0.93 ± 0.1	-0.94 ± 0.1	0.75 ± 0.1	0.76 ± 0.1	0.75 ± 0.1	0.76 ± 0.1	1250 ± 23	1248 ± 23	1250 ± 23	1248 ± 23	1250 ± 23	1248 ± 23
14,15-EET	4	-0.82 ± 0.1	-0.84 ± 0.1	0.66 ± 0.1	0.67 ± 0.1	0.66 ± 0.1	0.67 ± 0.1	1253 ± 21	1254 ± 21	1253 ± 21	1254 ± 21	1253 ± 21	1254 ± 21

Effect of the four EET regioisomers (each 0.3 μM) on potential difference (PD), short circuit current (I_{sc}) and transepithelial resistance (R_{TE}) of MDCK C7 cells mounted in Ussing-type chambers (mean ± SE). Except for the 5,6-EET, all EETs including 5,6-EET methyl ester (5,6-EET-Me, 0.3 μM) and the 5,6-dihydroxyicosatrienoic acid (5,6-DHET, 0.3 μM) were added to the apical bath solution (*, p<0.05 vs. basal, [†], p<0.05 vs. apical 5,6-EET). Note that the 5,6-EET is only effective on the apical (luminal) membrane.

Table 3

Effect of 5,6-EET metabolites on MDCK C7 monolayers.

agonist	n	basal	experiment	basal	experiment	basal	experiment
			PD (mV)		I _{sc} (μA/cm ²)		R _{TE} (Ω·cm ²)
5,6-epoxy-PGE ₁	3	-0.51 ± 0.3	-3.7 ± 0.2*	0.63 ± 0.2	2.84 ± 0.3*	1321 ± 52	1287 ± 46
5,6-dihydroxy-PGE ₁	3	-0.8 ± 0.1	-1.2 ± 0.4	1.08 ± 0.2	1.41 ± 0.3	1215 ± 37	1216 ± 35

Effect of 5,6-EET metabolites on potential difference (PD), short circuit current (I_{sc}) and transepithelial resistance (R_{TE}) of MDCK C7 cells mounted in Ussing-type chambers (mean ± SE). The substances were added to the apical bath solution (*, p<0.05 vs. basal).