Short-Term Regulation of Basolateral Organic Anion Uptake in Proximal Tubular OK cells: EGF Acts via MAPK, PLA₂, and COX1

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Abstract. The organic anion transport system of the kidney is of major importance for the excretion of a variety of endogenous compounds, drugs, and potentially toxic substances. The basolateral uptake into proximal tubular cells is mediated by a tertiary active transport system. Epidermal growth factor (EGF) leads to an increase in the basolateral uptake rate of the model substrate para-aminohippuric acid (PAH) in opossum kidney (OK) cells. This stimulation is mediated by successive activation of the mitogen-activated protein kinases, mitogen-activated/extracellular signal-regulated kinase kinase (MEK) and extracellular regulated kinase isofoms 1 and 2 (ERK1/2). This study investigates the regulatory network of EGF action on PAH uptake downstream ERK1/2 in more detail. EGF stimulation of the basolateral uptake rate of [14C]PAH was abolished by the phospholipase A₂ inhibitor AACOCF3. [14C]PAH uptake was enhanced by arachidonic acid. Furthermore, EGF led to an increase in arachidonic acid release and to the generation of prostaglandins. AACOCF3 did not influence EGF-induced ERK1/2 activation, indicating that ERK1/2 is upstream of PLA₂. In addition, EGF stimulated the influx of extracellular Ca²⁺. However, Ca²⁺-influx was not required for the stimulatory action of EGF on [14C]PAH uptake. Inhibitors of COX and lipoxygenases reduced [14C]PAH uptake dose-dependently, whereas inhibition of cytochrome P450 did not. In the presence of indomethacin, EGF had no stimulatory effect on [14C]PAH uptake. The inhibitory effect of indomethacin was not due to competitive action on PAH uptake. Furthermore, prostaglandin E₂ (PGE₂) increased basolateral [14C]PAH uptake rate dose-dependently, and this increase was also observed in the presence of indomethacin. Selective inhibition of COX2 by indomethacin amid or indomethacin n-heptyl ester did not inhibit [14C]PAH uptake, whereas selective inhibition of COX1 dose-dependently inhibited [14C]PAH uptake. This and previous data lead to the conclusion that EGF successively activates MEK, ERK1/2, and PLA₂, leading to an increased release of arachidonic acid. Subsequently, arachidonic acid is metabolized to prostaglandins via COX1, which then mediate EGF-induced stimulation of basolateral organic anion uptake rate.

The organic anion transport system of the renal proximal tubule plays a crucial role in the excretion of a variety of potentially toxic compounds (1). This system consists of a basolaterally located organic anion exchanger and a less well characterized transport step at the apical membrane (2).

The basolateral organic anion exchanger is a tertiary active transport system, dependent on an inward-directed Na⁺-gradient to drive the uptake of α-ketoglutarate, which is then exchanged for organic anions (1–4). The basolateral exchanger for organic anions and dicarboxylates was cloned by three independent groups (5–7) in 1997 and named OAT1 (rat), ROAT1 (rat), or rROAT1 (winter flounder). The homologous protein was cloned from human kidney only recently and was called hOAT1 (8) or hPAHT (9,10). The genomic DNA from human hOAT1 is organized in ten exons, and up to four isoforms are described (11). Furthermore, it was shown that OAT1 represents the basolateral, polyspecific transporter for organic anions (12), which has been functionally described (4). Meanwhile, three additional homologues were cloned and called OAT2 (13), OAT3 (14), and OAT4 (15). These clones show 40% homology in amino acid sequence compared with OAT1, and they differ from OAT1 in substrate specificity and expression pattern. Furthermore, these proteins are not exchangers like OAT1, but seem to work as facilitators (16).

Little is yet known about the modulation of this transport system. Nagai et al. (17) showed an inhibition of basolateral uptake and secretion of organic anions in opossum kidney (OK) cells by parathyroid hormone via a staurosporine-sensitive mechanism. Inhibition of basolateral organic anion transport by stimulation of protein kinase C (PKC) was reported in isolated tubules of killifish (18). rOAT3 from rat is also inhibitable by PKC (19), and mOAT from mouse is regulated by PKC and phosphatases (20). The basolateral exchanger of organic anions and dicarboxylates in isolated proximal tubules of rabbit kidney was shown to be regulated by Ca²⁺/calmodulin-dependent protein kinase II, thyroxin kinase, phosphatidylinositol-3-kinase, and mitogen-activated protein kinases.
C. The concentrations of the radio-labeled substrates applied were measured in phosphate-buffered ringer at pH 7.4. Inhibition of the net secretory transport of organic anions by bradykinin and phenylephrine via PKC was shown in isolated perfused rabbit proximal tubules (23). Recently, You et al. (20) showed that PKC inhibits murine OAT without direct phosphorylation of the transport protein itself.

As we could show only recently, epidermal growth factor (EGF) stimulates the basolateral organic anion transport via MAPK (24). We therefore investigated this regulatory pathway in more detail. For this purpose, we chose the proximal tubule-derived OK cell line cultured on permeable supports, a well-characterized model system to investigate organic anion secretion (24,25).

In this study, we investigated the mechanisms of the acute effect of EGF on basolateral uptake of para-aminohippuric acid (PAH) in OK cells. As described in reference 24, EGF leads to successive activation of the MAPK, ERK kinase (MEK), and extracellular regulated kinase 1/2 (ERK1/2). We herein present data indicating that ERK1/2 then stimulates PLAX, leading to an increase in release of arachidonic acid, which is metabolized to prostaglandins via COX1. Finally, prostaglandin E2 (PGE2) is shown to stimulate the basolateral uptake of PAH in OK cells.

Materials and Methods

Cell Culture

OK cells were obtained from Dr. Jörg Biber, Department of Physiology, University of Zurich. Cells were maintained in culture at 37°C in a humidified 5% CO2, 95% air atmosphere. The growth medium was minimal essential medium (MEM), pH 7.4, supplemented with Earl salts, nonessential amino acids, 10% (vol/vol) fetal calf serum (FCS) and 26 mmol/L NaHCO3. Cells were cultured on permeable supports (pore diameter, 3 μm; Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) for transport measurements. The effective growth area on one permeable support was 4.3 cm2/filter. All studies were performed between passage 60 and 100. The seeding density was 0.4-106 cells/cm2. The medium was changed every third day, and the medium was changed every 2 s.

Measurement of Cytosolic Free Ca2+

Cytosolic free Ca2+ ([Ca2+]i) was determined using the Ca2+-sensitive dye fura-2 AM as described in detail elsewhere (26). In brief, after serum depletion for 24 h, cells were incubated with PBS containing fura-2 AM in a final concentration of 5 μmol/L for 15 min. The coverslips were subsequently mounted on the stage of an inverted Axiovert 100 TV microscope (40× magnification, oil immersion; Zeiss, Göttingen, Germany). The fluorescence signal was monitored at 510 nm with the excitation wavelength alternating between 334 and 380 nm using a 100 W xenon lamp and an automatic filter change device (Zeiss, Göttingen, Germany). The fluorescence signal was monitored at 510 nm with the excitation wavelength alternating between 334 and 380 nm using a 100 W xenon lamp and an automatic filter change device (Zeiss, Göttingen, Germany). The sampling rate was one ratio every 2 s. [Ca2+]i was calculated according to reference 26 assuming a dissociation constant (Kd) of 225 nmol/L, after subtraction of background fluorescence. The maximum and minimum ratios (Rmax and Rmin, respectively) were measured after addition of calibration solutions containing 1 mmol/L Ionomycin and 1 mmol/L Ca2+ (Rmax) or 1 mmol/L ethylene glycol-bis[β-aminoethyl ether]-N,N,N',N'-tetraacetic acid (EGTA) and no Ca2+ (Rmin).

Arachidonic Acid Release Assay

OK cells were seeded on six-well plates and cultivated until confluence. Cells were incubated with 20000 Bq of [3H]-labeled arachidonic acid for 12 h, leading to incorporation rates of approximately 85%. After washing the cells 6 times with ice-cold PBS, cells were incubated with 2 ml of PBS containing the substances mentioned in the results section at 37°C for 10 min. After that, apical media was collected in the transport buffer, the cells were washed twice with ice-cold PBS. The filters containing the cells were subsequently washed twice with ice-cold PBS and cut from the supports. Radioactivity of the cells was measured using a liquid scintillation counter (Packard Instruments, Frankfurt, Germany). Counts of cells on filters were corrected for nonspecific binding on filters by subtraction.

Western Blot Analyses

OK cells were rinsed three times with PBS followed by a 10-min incubation with EGF and/or AACOCF3. Cells were subsequently washed with ice-cold PBS three times and lysed in ice-cold Triton X-100 lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 40 mM β-glycerophosphate, 200 μM sodium orthovanadate, 0.1 mM phenylmethylsulfonflury fluoride, 1 μg/ml leupeptin, 1 μM pepstatin A, 1% Triton X-100) for 25 min at 4°C. Insoluble material was removed by centrifugation at 12000 × g for 15 min at 4°C. The protein content was determined using a microbicinchoninic acid assay (Pierce, Rockford, IL) with bovine serum albumin (BSA) as a standard. Cell lysates were matched for protein, separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride microporous membrane. Membranes were subsequently blotted with rabbit anti-PERK1/2 (p42/p44) antibody (New England Biolabs, Beverly, MA). The primary antibody was detected using horseradish peroxidase–conjugated goat anti-rabbit IgG, and visualized by ECL. Western blotting reagents and Hyperfilm ECL (Amersham Life Sciences International, Buckinghamshire, England). According to the manufacturer’s handbook, Hyperfilm ECL exhibits a linear response to the light produced from enhanced chemiluminescence. Additionally, linearity was verified for our experimental conditions by a dilution series with increasing amounts of total cell protein (24). Western blotting was performed with protein from three independent extractions from three independent cell culture passages. Blots were analyzed using SigmaGel 2000 Software (Jandel Scientific, Rafael, CA).
collected and 0.5 ml of 1 N NaOH was added to lyse the cells. Apical media and lysate were counted in a liquid scintillation counter (Packard Instruments, Frankfurt, Germany). Arachidonic acid release was calculated as amount of radioactivity released in % of total radioactivity present in the cells at the beginning of incubation. The release of radioactivity was due to PLA₂ action and is a measure for arachidonic acid release, as indicated by HPLC data.

Measurement of Arachidonic Acid Metabolism by HPLC

HPLC was performed as described in references 27–29. In brief, OK cells were seeded on six-well plates and cultivated until confluence. Cells were incubated with 20000 Bq of [³H]-labeled arachidonic acid for 12 h, leading to incorporation rates of around 85%. After washing the cells 6 times with ice-cold PBS, cells were incubated with 2 ml of PBS containing the substances mentioned in the results section at 37°C for 10 min. Apical media was collected and the CCl₄-soluble phase was extracted (vol, 1:1). CCl₄ was evaporated under nitrogen atmosphere, and the pellet was resolved in HPLC mobile phase buffer. Arachidonic acid and its metabolites were separated by HPLC with an Ultrasphere C18 column (Beckmann Instruments). Metabolites were identified by co-elution with authentic standards. [³H]-labeled arachidonic acid standard was detected by fractionation and scintillation counting. PGE₂ standard was detected by spectrometry at a wavelength of 235 nm. The mobile phase consisted of methanol–water–acetic acid (75:24.95:0.05; vol:vol:vol) (pH 5.4) and a flow rate of 1 ml/min. Fractions of 1 ml were collected and counted in a liquid scintillation counter (Packard Instruments, Frankfurt, Germany).

Statistical Analyses

Data are presented as mean ± SEM. The number of culture plates or filters used to perform the measurements (n) is given in the text or in the figures. Statistical significance was determined by unpaired t test or ANOVA as appropriate. Results were considered statistically different at P < 0.05. Significant difference is indicated by asterisks.

Materials

[¹⁴C]PAH, [³H]mannitol, and [³H]arachidonic acid were purchased from American Radiolabeled Chemicals Inc., St. Louis, MO. PD98059 was from Alexis Corp., Läufelfingen, Switzerland. Indomethacin amyd was from Calbiochem, San Diego, CA. Antibody against the phosphorylated form of ERK1/2 (pERK1/2) was from New England Biolabs Inc., Beverly, MA. If not stated otherwise, all other chemicals were from Sigma. EGF from Sigma was used as human, recombinant substance.

Results

Arachidonic Acid Is Involved in EGF-Mediated Stimulation

EGF stimulation of initial basolateral uptake of [¹⁴C]PAH was completely abolished by the compound AACOCF₃ (25 μM), an inhibitor of PLA₂ (Figure 1A). AACOCF₃ alone exerted no effect on the basolateral [¹⁴C]PAH uptake rate. This indicates a regulatory role for arachidonic acid, the product of PLA₂-activity, on EGF-stimulated initial basolateral uptake of [¹⁴C]PAH in proximal tubular OK cells. As shown in Figure 1B, preincubation with arachidonic acid (30 μM for 10 min) doubled initial basolateral uptake rate of [¹⁴C]PAH in OK cells, whereas no competitive interaction of arachidonic acid and [¹⁴C]PAH was detectable.

EGF Leads to Increased Arachidonic Acid Release

As arachidonic acid seemed to be involved in EGF action on [¹⁴C]PAH uptake, we investigated the effect of EGF on arachidonic acid release in OK cells. EGF (10 ng/ml) increased arachidonic acid release after 10 min, as indicated by results of the [³H]arachidonic acid release assay shown in Figure 2A. The EGF-mediated stimulation of [³H]arachidonic acid release was completely inhibited by AACOCF₃ (inhibitor of PLA₂).
AACOCF3 alone had no effect on \([^{3}H]\)arachidonic acid release. These data indicate that EGF stimulates PLA2, which then leads to an increase of arachidonic acid release. Using the HPLC technique described in the Materials and Methods section, we could show that 50% of the radioactivity released by EGF is \([^{3}H]\)arachidonic acid and thus validated the \([^{3}H]\)arachidonic acid release assay (Figure 2B).

**Arachidonic Acid Metabolites Are of the Prostaglandin Type**

Additionally, we could show that the detectable arachidonic acid metabolites are of the prostaglandin type, as around 20% of the EGF-released \([^{3}H]\) counts co-elute in the range of prostaglandins as indicated by the PGE\(_2\) standard and references 27–29. According to the literature mentioned, leukotrienes or metabolites of the icosatetraenoic acid type appear as peaks between the arachidonic acid and the prostaglandin fraction under our HPLC conditions. As no such peaks were detected, EGF does not lead to a release of leukotrienes or metabolites of the icosatetraenoic acid type during 10 min in OK cells (Figure 2B).

**EGF Activation of PLA2 is Abolished by Inhibition of MEK**

As we have shown previously (24), EGF successively stimulates MEK and ERK1/2 in OK cells. It is known that ERK1/2 activate PLA2 (30); we therefore investigated whether inhibition of ERK1/2 phosphorylation also inhibits arachidonic acid release in OK cells. As shown in Figure 2A, EGF-mediated \([^{3}H]\)arachidonic acid release was completely prevented by 5 \(\mu M\) PD98059 (specific inhibitor of MEK), whereas 5 \(\mu M\) PD98059 alone had no effect. Thus, ERK1/2 phosphorylation is necessary for the EGF-mediated increase in PLA2 activity in proximal tubular OK cells.

**EGF-Mediated ERK1/2 Activation Is Not Altered by PLA2 Inhibition**

The above data are evidence that PLA2 is activated by the MEK–ERK1/2 pathway; we therefore investigated whether PLA2 has any influence on ERK1/2 activity. Figure 3 shows that EGF-mediated activation of ERK1/2 is not affected by inhibition of PLA2 with 25 \(\mu M\) AACOCF3. Thus, ERK1/2 is upstream of PLA2, and no feed back mechanism on the ERK1/2 pathway exists downstream PLA2 in proximal tubular OK cells.

**EGF Leads To Influx of Ca\(^{2+}\)**

EGF is known to acutely increase intracellular free Ca\(^{2+}\) concentration in proximal tubular cells via an increased influx of extracellular Ca\(^{2+}\) (31). Thus, we investigated the effect of EGF on the intracellular free Ca\(^{2+}\) concentration in OK cells. As shown in Figure 4A, EGF led to a slight increase of intracellular free Ca\(^{2+}\) from approximately 80 nM to approximately 110 nM. The Ca\(^{2+}\) concentrations measured in OK cells correspond to already published data from our lab (32). To investigate whether the increase of Ca\(^{2+}\) is due to release from intracellular stores or to increased influx, we investigated the effect of EGF in the presence of low extracellular Ca\(^{2+}\). As shown in Figure 4B, the EGF-induced increase of intracellular free Ca\(^{2+}\) was not present under this conditions. Addition of Ca\(^{2+}\) to the extracellular bath restored EGF-mediated increase

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**Figure 2.** Effect of epidermal growth factor (EGF; 10min; 10 ng/ml) on arachidonic acid (AA) release in OK cells. (A) Effect of EGF and/or inhibitors of mitogen-activated/extracellular signal-regulated kinase kinase (MEK) (PD98059) or PLA2 (AACOCF3) on the release of \([^{3}H]\)-labeled AA in OK cells. For incorporation and subsequent release of \([^{3}H]\)-labeled AA, OK cells were treated as mentioned in the Materials and Methods section. The cells were incubated with EGF alone (10 ng/ml) and in the presence of PD98059 (5 \(\mu M\)) or AACOCF3 (25 \(\mu M\)). Reference values are \([^{3}H]\) release in untreated controls and \([^{3}H]\) release in the presence of PD98059 alone or AACOCF3 alone, respectively. n = 5 for every bar. * P < 0.05 versus control. (B) Representative HPLC tracing of CCl\(_4\)-soluble \([^{3}H]\) counts in the supernatant of controls and OK cells treated with EGF (10 ng/ml) for 10 min. Cells and supernatants were treated as described in the Materials and Methods section. HPLC fractions were collected and scintillation counted. Data are presented as % of total \([^{3}H]\)-counts of control. HPLC tracing of control cells after 10 min is presented as gray line; HPLC tracing of OK cells treated with EGF (10 ng/ml) for 10 min is presented as black line. Retention time of AA and prostaglandin E\(_2\) (PGE\(_2\)) were determined according to the Materials and Methods section.
of intracellular free Ca$^{2+}$). Thus, in accordance with reference 31, the EGF-induced increase in intracellular free Ca$^{2+}$ is due to an increased influx of extracellular Ca$^{2+}$.

**EGF Stimulation Persists in Low Extracellular Ca$^{2+}$**

Intracellular Ca$^{2+}$ is known to influence a number of regulatory enzymes such as isoforms of PLA$_2$ (33); we therefore investigated whether EGF stimulates basolateral organic anion uptake when Ca$^{2+}$-influx is prevented. As shown in Figure 5, lowering extracellular Ca$^{2+}$ led to a decreased uptake rate of

$[^{14}C]$PAH. However, EGF still stimulated initial basolateral uptake under these conditions. Thus, the increase of intracellular free Ca$^{2+}$ by EGF is not involved in EGF-mediated stimulation of initial basolateral uptake of PAH.

**COX Metabolites Mediate EGF Stimulation of Organic Anion Uptake**

In the kidney, three major pathways for processing of arachidonic acid are described. Cyclooxygenase (COX), lipoygenase, and cytochrome P450 are the major components of these (34). Inhibition of cytochrome P450 activity by increasing concentrations of 17-octadecynoic acid (17-ODYA; IC$_{50}$ = 5 to 7 $\mu$M; [29]) did not influence the initial basolateral PAH uptake rate as shown in Figure 6A. In contrast, inhibition of lipoygenase by nordihydroguaiaretic acid (Figure 6B; NDGA, IC$_{50}$ = approximately 50$\mu$M [35]) affected PAH uptake only at 100 $\mu$M but not at lower concentrations. This effect of

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**Figure 3.** Effect of EGF and/or AACOCF3 on phosphorylation of extracellular-regulated kinase 1/2 (ERK1/2) in OK cells. Western blot of OK cell protein with anti-pERK1/2 antibody, according to the Materials and Methods section. Cells were treated with 10 ng/ml EGF and/or 25 $\mu$M AACOCF3 for 10 min before protein was extracted as described. Amount of total OK cell protein per lane was 20 $\mu$g. (A) Histogram of three blots from three different protein extractions and cell culture passages of OK cells. EGF leads to a significant stimulation of pERK1/2 amount. Protein extracts from cells treated with AACOCF3 are not different from controls. Protein extracts from cells treated with AACOCF3 in combination with EGF do not differ from EGF alone. * $P < 0.05$ versus control. (B) Single most typical Western blot showing the effect of EGF and/or PD98059 on phosphorylation of ERK1/2 in OK cells. The antibody used leads to a specific staining of two protein bands at 42 kD and 44 kD representing pERK1 and pERK2. No other protein signals were detected.

**Figure 4.** Effect of EGF (10 ng/ml) on intracellular free Ca$^{2+}$ in OK cells. (A) Intracellular Ca$^{2+}$ concentration in OK cells after addition of 10 ng/ml EGF to the extracellular bathing solution. Representative tracing showing the response to EGF; $n = 20$. (B) Representative tracing showing the effect of extracellular Ca$^{2+}$ on the EGF (10 ng/ml)-induced increase in the fluorescence ratio as a measure of intracellular free Ca$^{2+}$. $n = 22$. 

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NDGA at high concentrations cannot be interpreted unequivocally in a way that lipoxgenases are involved, due to the lack of specificity of NDGA at these high concentrations. As in this concentration range, NDGA is also an inhibitor of COX (36). In contrast, inhibition of COX by indomethacin (Figure 6C; IC$_{50}$ approximately 1 M according to manufacturer) at low concentrations completely abolished basolateral PAH uptake. As shown in Figure 6C, the inhibition by indomethacin was dose-dependent with an IC$_{50}$ value of approximately 1 M.

Because our HPLC data show that only COX metabolites are produced after EGF incubation, prostaglandins are the only candidates for mediating the effect of EGF; we therefore investigated the role of COX in more detail.

EGF Stimulation of Basolateral PAH Uptake Rate is Abolished by Inhibition of COX

As COX turned out to be involved in regulation of basolateral uptake of PAH, we investigated the role of COX in EGF mediated stimulation of basolateral organic anion uptake. Figure 7A shows that inhibition of COX by 1 M indomethacin completely inhibits PAH uptake in the presence of arachidonic acid. Figure 7B again shows the stimulatory action of EGF (10 ng/ml) on initial basolateral uptake rate of PAH. This stimulation is completely abolished in the presence of indomethacin (1 M). Inhibition of COX can abolish the action of arachidonic acid, which is known to be released after EGF. Thus, COX activity is prerequisite for EGF-induced stimulation of initial basolateral uptake of PAH in OK cells.

COX1 Is the Relevant Isoform in PAH Uptake Regulation

To determine which COX isoform is involved in the regulation of basolateral PAH uptake in OK cells, we investigated the effect of indomethacin amid and indomethacin n-heptyl ester, both specific inhibitors of COX2. Although an IC$_{50}$ value of 40 nM was stated by the manufacturer for both substances, neither 100 nM nor 1000 nM indomethacin amid or indomethacin n-heptyl ester inhibited the initial basolateral uptake of PAH in OK cells (Figure 8, A and B). Thus, COX2 does not seem to be involved in short-term regulation of PAH uptake. As shown
in Figure 8C, SC-560, a specific inhibitor of COX1 (IC$_{50}$ = 9 nM), dose-dependently inhibited initial basolateral PAH uptake. Thus, COX1 is involved in short-term regulation of PAH uptake in OK cells.

**Discussion**

Basolateral uptake of organic anions into proximal tubular epithelial cells occurs against the electrochemical gradient. This transport step is mediated by a polyspecific, tertiary active organic anion transport system. As already mentioned in the introduction, little is known about the regulation of the basolateral uptake step of organic anion secretory transport. In a previous study, we reported the acute stimulation of initial basolateral organic anions in OK cells by EGF (31). This
the COX inhibitor indomethacin. Cells were incubated with 0.1 H9262 bars. * Pn before PAH uptake was determined.

Figure 9. Effect of PGE 2 on initial basolateral uptake of 10 µM [14C]PAH in OK cells. (A) Effect of 10 nM or 100 nM PGE 2 on initial basolateral PAH uptake. Cells were incubated with the respective concentration of PGE 2 for 10 min before PAH uptake was determined. n is shown in brackets within the bars. * P < 0.05 versus the respective control. (B) Effect of PGE 2 in the absence or presence of the COX inhibitor indomethacin. Cells were incubated with 0.1 µM PGE 2 or 1 µM indomethacin alone or in combination for 10 min before PAH uptake was determined. n is shown in brackets within the bars. * P < 0.05 versus the respective control.

Figure 9. Effect of PGE 2 on initial basolateral uptake of 10 µM [14C]PAH in OK cells. (A) Effect of 10 nM or 100 nM PGE 2 on initial basolateral PAH uptake. Cells were incubated with the respective concentration of PGE 2 for 10 min before PAH uptake was determined. n is shown in brackets within the bars. * P < 0.05 versus the respective control. (B) Effect of PGE 2 in the absence or presence of the COX inhibitor indomethacin. Cells were incubated with 0.1 µM PGE 2 or 1 µM indomethacin alone or in combination for 10 min before PAH uptake was determined. n is shown in brackets within the bars. * P < 0.05 versus the respective control.

stimulation was shown to be mediated by successive activation of MEK and ERK1/2. In this study, we investigated the regulatory network downstream ERK1/2 in more detail.

Stimulation of PLA 2 has been described to occur by EGF and MAPK (30–39). We therefore investigated whether the EGF effect on organic anion uptake is mediated via PLA 2. As shown in the Results section, inhibition of PLA 2 inhibits both EGF-induced stimulation of the basolateral uptake rate of PAH and EGF-mediated release of arachidonic acid as indicated by [3H]arachidonic acid release assay and HPLC. Arachidonic acid release was also prevented by inhibition of ERK1/2 phosphorylation. Furthermore, incubation of OK cells with arachidonic acid increased the basolateral uptake rate of PAH, whereas no competitive interaction of arachidonic acid with PAH uptake was detected. Taken together with the data already presented in reference 24, which shows the successive activation of MEK and ERK1/2, we consider this as evidence that the acute EGF action on basolateral organic anion transport in OK cells is mediated via successive activation of MEK, ERK1/2, and then PLA 2.

We already mentioned that it is well known that EGF leads to an acute increase of Ca 2+ influx in proximal tubular cells. Because intracellular Ca 2+ influences a variety of signaling steps, such as Ca 2+-sensitive isoforms of PLA 2 (33,39), we investigated the role of changes in cytosolic Ca 2+ in our experimental setup. The fact that low extracellular Ca 2+ decreases intracellular free Ca 2+ and the basolateral uptake rate to 40% as compared with control indicates a certain Ca 2+-dependency of constitutive basolateral PAH uptake. By contrast, the stimulatory effect of EGF on PAH uptake was not prevented by low Ca 2+ conditions. Although the absolute EGF-induced transport was slightly reduced, the relative stimulation elicited by EGF was in the same range under low Ca 2+ conditions as compared with controls (68% compared with 64% of the respective control value). Thus, the action of EGF on PAH uptake is independent of Ca 2+ under our conditions.

As already mentioned, three major pathways of arachidonic acid oxidative metabolism are described, including the key enzymes COX, lipoxygenase, and cytochrome P450. Inhibitors of COX and lipoxygenase, but not of cytochrome P450, completely abolished basolateral PAH uptake rate in OK cells. Due to our own experiments (data not shown), a competitive action of NDGA up to 100 µM is excluded. Salari et al. (40) investigated the action of NDGA on the generation of lipoxygenase products in a whole cell system. After 20 min, the metabolites were detected by HPLC. Their study shows that NDGA inhibits its 5-, 12-, and 15-lipoxygenase in a whole cell system with increasing IC 50 values (approximately 10 -7 M, 10 -6 M, and 3 × 10 -5 M). Thus, the NDGA concentrations used were suitable to inhibit lipoxygenase(s). However, the lipoxygenase inhibitor was effective only at high concentrations with limited specificity. As no lipoxygenase products (leukotriene peaks) were detected in HPLC of OK cells treated with EGF, lipoxygenases are not involved in processing of arachidonic acid released due to EGF action. As measured by Lin et al. (41), 17-ODYA inhibits cytochrome P450 activity in rat proximal tubule primary culture cells with an IC 50 of about 2.5 µM (by HPLC and GC-MS). In the same article, the authors show that no EGF-inducible cytochrome P450 activity was detectable in OK cells. The above-mentioned IC 50 of 17-ODYA in proximal tubular cells is similar to the IC 50 in rat renal cortical microsomal fractions of approximately 5 µM, as measured by Wang et al. (29). Thus, the concentrations of 17-ODYA used are sufficient to inhibit cytochrome P450 activity. In addition, we have evidence from the literature that EGF does not lead to release of cytochrome P450 products in OK cells. This is in agreement with our own HPLC data. Thus, the participation of the cytochrome P450 pathway is ruled out. Thus, prostaglandins are the only metabolites that emerged after EGF exposure as detected by HPLC. Additionally, COX inhibition completely abolished EGF stimulation of PAH uptake. In summary, the action of EGF on PAH uptake is mediated by COX and not by lipoxygenase or cytochrome P450. This does not

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rule out that leukotrienes may have an effect on organic anion uptake in general.

It is well known that renal cortex tissue (42), renal epithelial cell lines (43), and isolated proximal tubules (44) produce PGE$_2$. The fact that inhibition of COX diminishes organic anion uptake in the OK cell system alone is, of course, not the final proof for EGF-stimulated PGE$_2$ formation. However, HPLC data show the appearance of a prostaglandin fraction after EGF preincubation, and the PGE$_2$ standard co-elutes with this prostaglandin fraction. This is evidence that PGE$_2$ is one COX metabolite released after EGF stimulation in OK cells. As PGE$_2$ is the major and most potent COX metabolite (38) in the kidney, we decided to focus on PGE$_2$ in the following experiments. PGE$_2$ enhanced the basolateral organic anion uptake rate, also when COX was inhibited. Furthermore, inhibition of COX completely abolished the stimulatory action of EGF. Taken together, these data are analytic and pharmacologic evidence for a contribution of PGE$_2$ to EGF-mediated stimulation of basolateral organic anion uptake. In addition, EGF-stimulated PGE$_2$ release has been described in proximal tubular cells before (45). We therefore conclude that EGF induces prostaglandin formation in OK cells and that PGE$_2$ is probably one of the active metabolites. Prostaglandins then increase basolateral organic anion uptake rate by a yet not known mechanism.

To functionally define the isoform of COX responsible for transport stimulation (prostaglandin formation), we investigated the effect of the selective COX2 inhibitors indomethacin amid and indomethacin n-heptyl ester and the selective COX1 inhibitor SC-560 in comparison with the nonspecific COX inhibitor indomethacin. In contrast to indomethacin, reasonable concentrations of indomethacin amid and indomethacin n-heptyl ester did not inhibit PAH uptake rate in OK cells at all. SC-560 showed a dose-dependent inhibition of PAH uptake. Thus, in OK cells under our conditions, prostaglandins are synthesized by COX1. This is in good agreement with the literature, which shows that production of prostaglandins in kidney cortex is predominantly mediated by COX1 (42). Additionally, inhibition of COX2 by celecoxib in humans did not affect pharmacokinetics of the organic anion methotrexate (46). Taken together, these data support our hypothesis that EGF exerts its acute action on PAH uptake via COX1. This seems reasonable, as COX2 represents the inducible form of COX and 10-min preincubation with EGF is too short to induce new proteins.

Indomethacin inhibits the organic anion uptake rate in a dose-dependent manner. Incubation with up to 300 µM indomethacin for up to 60 min is reported not to cause nonspecific toxic actions in proximal tubular cells (47). Thus, nonspecific toxic actions do not explain the observed effects. Indomethacin is a substrate for the organic anion transporter; the observed inhibition of the organic anion uptake rate could therefore be due to a competitive action (48). For a competitive inhibition to occur, the crucial parameter is the extracellular concentration of the competing substance (indomethacin). As we have shown previously, the washing procedure used reduces the extracellular concentration of incubation agents about by four to five orders of magnitude (49). Thus, extracellular indomethacin concentration after preincubation, even with 100 µM, is reduced to approximately 1 to 10 nM by the washing procedure. Yet, the half-maximal inhibitory concentration for cis-inhibition of PAH transport by indomethacin is approximately 10 µM (50). Thus, the extracellular concentration of indomethacin is too low to competitively inhibit PAH uptake. In addition, if the action of indomethacin was not due to its properties as a COX inhibitor but due to its properties as a substrate, it should trans-stimulate PAH uptake after preincubation as a result of the above-mentioned accumulation. This was clearly not the case. We therefore interpret our data in a way that indomethacin inhibition in our specific setup is due to its COX-inhibiting properties. Maybe even more importantly, we investigated the effect of the specific COX-1 inhibitor SC-560. One micromolar SC-560 also completely abolishes the stimulation of organic anion uptake by arachidonic acid while showing no competitive interaction with initial basolateral organic anion uptake in OK cells (data not shown). In summary, in our experimental setup, the inhibitory effect of COX-inhibitors (after 10 min) on arachidonic acid–mediated stimulation of organic anion uptake is due to their COX-inhibiting properties and not due to a direct interaction with organic anion transporters. The fact that PGE$_2$ stimulates PAH transport in the presence of indomethacin is additional evidence for our hypothesis.

Although their intrarenal distribution is only partially characterized, it is known that the kidney expresses four isoforms of E-prostanoid receptors (EP1–4) (37). Prostaglandins formed in response to EGF might therefore exert their action via one of these receptors. We must, however, remember that prostaglandins are also substrates for the basolateral organic anion transport system (44,50). Thus, prostaglandins formed in response to EGF might stimulate PAH uptake as trans-stimulating substrates. We do not know which of the two mechanisms applies for the stimulatory action of prostaglandins on organic anion uptake. Future experiments will have to investigate this issue in further detail. In fact, unpublished results from our laboratory show that PGE$_2$ stimulates PAH uptake even when maximal trans-stimulation with glutarate occurs. We therefore favor the hypothesis that the prostaglandin action is due to its properties as a auto- or parakrine signal substance. This is additional evidence for our idea that indomethacin reduces PAH uptake also due to its COX-inhibiting properties. Future experiments using prostanoid receptor antagonists will test this hypothesis.

According to previously published data (24) and to the data presented herein, we propose the following model for acute EGF stimulation of basolateral uptake in OK cells (Figure 10). EGF successively activates MEK, ERK1/2, and PLA$_2$, leading to an increased release of arachidonic acid, which is then metabolized to prostaglandins via COX1. PGE$_2$ is probably one of them. A similar signaling pathway was described for the bradykinin action in airway smooth muscle cells (51). The precise mechanism by which PGE$_2$ interacts with basolateral uptake of organic anions has to be investigated in future experiments, using selective prostanoid receptor agonists or antagonists.
as shown previously. ERK1/2 then activates PLA2, increasing the release of AA. AA is then metabolized into prostaglandin (PG, most probably PGE2), which then stimulates initial organic anion uptake by an as yet unknown mechanism.

As the organic anion transport system of the renal proximal tubule plays a crucial role in the excretion of a variety of potentially toxic compounds and drugs, the data presented here have pathophysiologic implications. The fact that COX inhibition decreases the basolateral step of organic anion secretion means that indomethacin influences its own excretion by inhibition of its own excretory pathway. Additionally, COX inhibitors will influence the tubular secretion of other substrates for the organic anion secretory system. Some case reports describe a loss of effectiveness of the diuretics acetazolamide or furosemide in patients under long-term COX-inhibitory therapy (52,53). As both diuretics reach the tubular lumen via the proximal tubular organic anion secretion system, their loss of function could be explained with the strong decrease of basolateral organic anion uptake rate due to COX inhibitors. The fact that the important substance class of COX inhibitors inhibits the basolateral step of organic anion transport is also important with respect to PAH-clearance measurements. This method is routinely used for the determination of renal blood flow and depends on a high and constant renal PAH extraction.

Acknowledgments

This work was supported by the Deutsche Forschungsgemeinschaft DFG grant Ge 905/3-4. We thank Prof. Dr. Stefan Silbernagl for stimulating and helpful discussions.

References


Figure 10. Summary showing the hypothesized model of the stimulation of the basolateral exchange step of PAH and dicarboxylates by EGF in OK cells. EGF stimulates the PAH/dicarboxylate exchanger via activation of the mitogen-activated protein kinases (MAPK), MEK and ERK1/2 as shown previously. ERK1/2 then activates PLA2, increasing the release of AA. AA is then metabolized into prostaglandin (PG, most probably PGE2), which then stimulates initial organic anion uptake by an as yet unknown mechanism.


