Action of EGF and PGE₂ on basolateral organic anion uptake in rabbit proximal renal tubules and hOAT1 expressed in human kidney epithelial cells


Submitted 9 September 2003; accepted in final form 21 November 2003

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Sauvant, C., D. Hesse, H. Holzinger, K. K. Evans, W. H. Dantzler, and M. Gekle. Action of EGF and PGE₂ on basolateral organic anion uptake in rabbit proximal renal tubules and hOAT1 expressed in human kidney epithelial cells. Am J Physiol Renal Physiol 286: F774–F783, 2004.—We recently showed that, in a proximal tubule cell line (opossum kidney cells), epithelial growth factor (EGF) stimulates basolateral organic anion transport (OAT) via ERK1/2, arachidonic acid, phospholipase A₂, and generation of prostaglandins. PGE₂ binds the prostanooid receptor and, thus, activates adenylate cyclase and PKA, which stimulate basolateral organic anion uptake. In the present study, we investigated whether this regulatory cascade is also true 1) for ex vivo conditions in isolated renal proximal (S2) tubules from rabbit and 2) in a human renal epithelial cell line stably expressing human OAT1 (IHKE-hOAT1). EGF activated ERK1/2 in S2 tubules and IHKE-hOAT1, and, in both cases, inhibition of ERK activation (by U-0126) abolished this stimulation. In S2 tubules and IHKE-hOAT1, EGF led to an increase of organic anion uptake, which again was inhibited by U-0126. PGE₂ stimulated basolateral organic anion uptake in rabbit S2 tubules and IHKE-hOAT1. EGF- and PGE₂-mediated stimulation of organic anion uptake was abolished by inhibition of PKA in rabbit S2 tubules and IHKE-hOAT1, respectively. We conclude that 1) stimulation of basolateral organic anion uptake by EGF or PGE₂ is a widespread (if not general) regulatory mechanism, 2) the signal transduction pathway involved seems to be general, 3) stimulation of basolateral organic anion uptake by EGF or PGE₂ is also present under ex vivo conditions and, thus, is not a cell culture artifact, 4) activation of OAT1 is sufficient to explain the stimulatory effects of EGF and PGE₂ in opossum kidney cells and rabbit S2 segments, and 5) stimulation of basolateral OAT1 by EGF or PGE₂ is also important in humans and, thus, may have clinical implications.

basolateral transport; epithelial growth factor; extracellular signal-regulated kinase 1/2; rabbit isolated proximal tubule; mitogen-activated protein kinase; mitogen-activated protein kinase kinase; IHKE cells; organic anion transport; phospholipase A₂; prostaglandin E₂; protein kinase A; regulation

THE ORGANIC ANION TRANSPORT (OAT) system of the renal proximal tubule plays a crucial role in the excretion of a variety of potentially toxic compounds (37, 56). This system consists of organic anion exchanger(s) located at the basolateral membrane and a less well-characterized transport step at the apical membrane (18).

The classical basolateral organic anion exchanger is the terminal step in a tertiary active transport system that is dependent on an inward-directed Na⁺ gradient to drive the uptake of α-ketoglutarate (α-KG), which is then exchanged for organic anions (17, 23, 38, 55). The basolateral exchanger for organic anions and dicarboxylates was cloned by three independent groups (46, 51, 58) in 1997 and named OAT1 (rat), ROAT1 (rat), and fROAT1 (winter flounder). Only recently, the homologous protein was cloned from human kidney and was called hOAT1 (13) or hPAHT (28, 34). The genomic DNA from human hOAT1 is organized into 10 exons, and 4 isoforms have been described (2). Furthermore, it has been shown that OAT1 represents the basolateral, polyspecific transporter for organic anions (45), which had been functionally described for some time (38). Meanwhile, three additional homologs have been cloned and called OAT2 (44), OAT3 (32), and OAT4 (10). These clones show 40% homology in amino acid sequence compared with OAT1, and they differ from OAT1 in substrate specificity and expression pattern. Furthermore, the original descriptions indicated that these proteins were not anion exchangers like OAT1 but seemed to work as facilitators of anion diffusion (43). Recently, however, new evidence has indicated that OAT3, which is located at the basolateral membrane, can also work as an appropriate exchanger for organic anions (50).

Little is known about the modulation of this transport system. Nagai and co-workers (36) showed an inhibition of basolateral uptake and secretion of organic anions in opossum kidney (OK) cells by parathyroid hormone via a stauroporine-sensitive mechanism. Inhibition of basolateral OAT by stimulation of protein kinase C (PKC) was reported in isolated tubules of killifish (35). ROAT3 from rat is also inhibitable by PKC (52), and mOAT (OAT1 from mouse) is regulated by PKC and phosphatases (60). 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, thyroxine kinase, phosphatidinositol-3-kinase, and mitogen-activated protein kinases (MAPK) (24). Furthermore, we showed an inhibition of initial basolateral organic anion uptake by bradykinin and phenylephrine via PKC in isolated rabbit proximal tubules (25). Inhibition of the net secretory transport of organic anions by bradykinin and phenylephrine via PKC was shown in isolated perfused rabbit proximal tubules (47). Recently, You and co-workers (60) showed that PKC inhibits murine OAT without direct phosphorylation of the transport protein itself.

As we showed recently in the proximal tubule cell line from opossum kidney (OK cells), epithelial growth factor (EGF) stimulates basolateral OAT via MAPK (39). We then presented...
data from this same cell line indicating that extracellular signal-regulated kinase 1/2 (ERK1/2) stimulates phospholipase A2 (PLA2), leading to an increase in release of arachidonic acid, which, via cyclooxygenase (COX)-1, is metabolized to prostaglandins. Prostaglandin E2 (PGE2) then stimulates the acid, which, via cyclooxygenase (COX)-1, is metabolized to PGE2. PGE2 acts via binding to a prostanoid receptor (most likely of the EP4 type), with subsequent activation of adenylyl cyclase and protein kinase A (PKA) (41).

In the present study, we addressed the following questions: 1) Is the stimulatory action of EGF also present in isolated renal proximal tubules under ex vivo conditions? 2) Can the effects of EGF be reproduced after expression of hOAT1 in a human renal epithelial cell line (IHKE)? 3) If so, is the mechanism of action in both cases dependent on ERK1/2 phosphorylation and/or PKA activation?

**MATERIALS AND METHODS**

**Cell Culture**

IHKE cells (obtained from Dr. S. Mollerup, National Institute of Occupational Health, Oslo, Norway) were cultured in DMEM-Ham’s F-12 medium (100 μl/cm² culture area) enriched with 13 mmol/l NaHCO₃, 15 mmol/l HEPES, 36 μg/ml hydrocortisone, 5 mg/ml human apotransferrin, 5 mg/ml bovine insulin, 10 μg/ml mouse EGF, 5 μg/ml sodium selenite, and 10% fetal calf serum, maintained at pH 7.3 and 37°C, and gassed with 95% O₂–5% CO₂. For transport measurements, the cells were cultured on permeable supports (3-μm pore diameter; Falcon, Becton Dickinson Labware). The effective growth area on one permeable support was 4.3 cm²/filter. All studies were performed between passages 40 and 80. The seeding density was 0.4 × 10⁶ cm⁻². The medium was changed every third day, and the monolayers were used for experiments at day 10 after seeding. All experiments were performed with cells that were serum starved for 24 h before the experiments.

**Stable Expression of hOAT1 in IHKE Cells**

hOAT1 was a generous gift from Dr. John B. Pritchard (National Institute of Environmental Health Sciences, Bethesda, MD). Briefly, IHKE cells were transfected with pcDNA3.1 containing hOAT1 using the Effectene transfection kit (Qiagen, Hilden, Germany) according to the manufacturer’s handbook. Resistance selection was performed with 600 mg/l G418 (Sigma, Taufkirchen, Germany). Transfection success was tested by measuring uptake of fluorescein (FL) into the transfected cell population. Subsequently, cell clones showing increased uptake of FL were selected by limited dilution cloning in 96-well plates. Several transporting clones were chosen, and p-aminohippurate (PAH)-inhibitable basolateral uptake of FL was measured. One of these clones was chosen and named IHKE-hOAT1 or IHKE-F10.

**RT-PCR**

RNA from cells was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). Briefly, RT-PCR was performed according to Superscript One-Step RT-PCR system protocol (Invitrogen). cDNA was generated at 55°C for 15 min, and the samples were denatured at 94°C for 2 min. PCR amplification was performed in 35 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 30 s. For hOAT1, the primers were 5’-CCC GCT GGC ACT CCT CCG GGA G-3’ (sense) and 5’-GTA GAG CTC GGC AGT CAT GCT CAC CA-3’ (antisense), covering bases 1072–1678 of hOAT1 cDNA and resulting in a 606-bp RT-PCR product.

**Measurements of OAT in Cultured Cells**

The volume of the apical and basolateral compartments were 1.3 and 2.5 ml to avoid hydrostatic pressure differences. Before each experiment, the cells were washed three times with phosphate-buffered Ringer solution containing (in mmol/l) 138 NaCl, 1 NaH₂PO₄, 4 Na₂HPO₄, 4 KCl, 1 MgCl₂, 1 CaCl₂, and 5 glucose and maintained at pH 7.4. Transport measurements were performed in PBS at pH 7.4 and 37°C.

OAT was always determined in the absence of possible stimulators or inhibitors by measuring the uptake of 10⁻⁶ M FL from the basolateral bath for a 3-min incubation period, according to a modified protocol of Cihlar and Ho (12). This FL uptake was recently described to be comparable to PAH uptake (41). At the end of the 3-min incubation, the cells were washed six times with ice-cold PBS until no FL was detectable in the washing solution. The cells were then lysed in 1 ml of 0.1% Triton X-100 in 20 mM MOPS, and fluorescence was counted in a multiwell-plate reader (Victor², Wallac Instruments). Counts were corrected by subtraction of FL uptake in the presence of 10 mM PAH. FL counts were normalized to lysate protein content as measured by bicinchoninic acid protein assay (Pierce, Rockford, IL).

To determine the effect of a possible stimulator on FL uptake, the cells were preincubated in a bath containing the stimulator for 10 min before uptake was measured. To investigate the effect of an inhibiting agent on the action of a stimulator, the following preincubation scheme was used: 1) inhibitor alone in the bath for 5 min followed by aspiration of the bath and replacement with inhibitor + stimulating agent in the bath for an additional 10 min, 2) inhibitor alone in the bath for 15 min, or 3) stimulator alone in the bath for 10 min.

**Preparation of Isolated Tubules**

New Zealand White rabbits were killed by intravenous injection of pentobarbital sodium. The kidneys were flushed via the renal artery with a solution containing 250 mM sucrose and 10 mM HEPES at pH 7.4. They were then removed gently and placed in chilled (4°C) medium for dissection. The standard solution used for dissecting and bathing the tubules contained (in mmol/l) 110 NaCl, 25 NaHCO₃, 5.0 KCl, 2.0 NaH₂PO₄, 1.0 MgSO₄, 1.8 CaCl₂, 10 sodium acetate, 8.3 d-glucose, 5.0 l-alanine, 0.9 glycine, 1.5 lactate, 1 malate, and 1 sodium citrate. This solution was gassed continuously with 95% O₂–5% CO₂ to maintain pH at 7.4. The osmolality of the solutions averaged 290 mosmol/kg H₂O.

Tubules were dissected from a slice of rabbit kidney without the aid of enzymatic agents, as described by others (8). All dissections were performed at 4°C, but all experiments were performed at 37°C. We used only proximal S2 segments in this study, because the S2 segment of the rabbit proximal tubule is the primary site of PAH secretion (59).

**Determination of Rate of Organic Anion Uptake Across the Basolateral Membrane of Intact Nonperfused Tubules**

These experiments were performed in a manner similar to that used previously (11, 21). Briefly, an appropriate number of tubule segments (4 for each condition to be studied) were teased from fresh renal tissue and maintained in oxygenated (95% O₂–5% CO₂) oil-covered Ringer solution at 4°C until the start of each experiment. The Ringer solution was warmed to 37°C for 5 min before the start of preincubation periods so that the tubules would be functioning at 37°C at the start of any treatment. In the present studies, tubules were preincubated at 37°C for 5 min before the measurement of uptake with one of the following four regimens: 1) control Ringer solution alone for 10 min, 2) Ringer solution containing possible stimulator (e.g., EGF) for 10 min, 3) Ringer solution containing possible inhibitor (e.g., U-0126) for 10 min, or 4) Ringer solution containing possible inhibitor (e.g., U-0126) for 10 min followed by Ringer solution containing possible stimulator (e.g., EGF) for 10 min more. At the end of the preincubation period, each tubule was transferred to a...
new bath with oil-covered incubation medium at 37°C containing ~6 μM [3H]PAH for 15 s. This time period was chosen because it is the shortest duration over which we can obtain adequate radioactive counting and, as demonstrated in our previous work (21), it is an appropriate duration for determination of the initial rate of uptake. No putative stimulators or inhibitors were present in the medium during these 15-s uptake measurements. The incubations were stopped by transferring each tubule into 10 μl of 1 N NaOH. The concentration of [3H]PAH in the cell water and the rate of uptake were determined as described below. Control and experimental uptakes were determined alternately and sequentially in tubules from the same kidney.

Determination of Cellular Concentration and Initial Rate of Uptake of PAH

The concentration of [3H]PAH in the cells was determined at the end of the uptake period by the method described in detail previously (15, 16, 19–21). Briefly, the tubule was pulled through the oil layer covering the bathing medium to minimize transfer of extracellular fluid and was immersed in 10 μl of 3% TCA for extraction of radioactivity. The activity of H extracted was determined by counting in a liquid scintillation system. The scintillation fluid was EcoLite (ICN Biomedicals, Irvine, CA) and water in a ratio of 1:5:1 (vol/vol).

Before the start of each experiment, we obtained an image of each tubule with a video camera, and the printed image was later used to determine the length and diameter of each tubule for calculation of the basolateral membrane area. Uptake was then standardized as moles per minute per square millimeter of basolateral membrane surface.

Western Blot Analysis

Cells or tubules were rinsed three times with PBS and then incubated as described for transport measurements in isolated rabbit tubules or IHHE-hOAT1 cells, respectively. Subsequently, cells or tubules were washed with ice-cold PBS three times and lysed in 10 μl of 3% TCA for extraction of proteins. The activity of H extracted was determined by counting in a liquid scintillation system. The scintillation fluid was EcoLite (ICN Biomedicals, Irvine, CA) and water in a ratio of 1:5:1 (vol/vol).

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Western Blot Analysis

Cells or tubules were rinsed three times with PBS and then incubated as described for transport measurements in isolated rabbit tubules or IHHE-hOAT1 cells, respectively. Subsequently, cells or tubules were 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 phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μM pepstatin A, and 1% Triton X-100) for 25 min at 4°C. Insoluble material was removed by centrifugation at 12,000 g for 15 min at 4°C. The protein content was determined using a microbicinchoninic acid assay (Pierce) with BSA as a standard. Cell or tubule lysates were matched for protein, separated by 12% SDS-PAGE, and transferred to a polyvinylidene difluoride microporous membrane. Subsequently, membranes were blotted with rabbit anti-phosphorylated ERK1/2 (pERK1/2, p42/p44) antibody (New England Biolabs, Beverly, MA). The primary antibody was detected using horseradish peroxidase-conjugated goat anti-antibody IgG and visualized by enhanced chemiluminescence Western blotting reagents and Hyperfilm ECL (Amersham Life Sciences International, Buckinghamshire, UK). 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 protein. Proteins were separated by 12% SDS-PAGE, transferred to a polyvinylidene difluoride microporous membrane. Subsequently, membranes were blotted with rabbit anti-phosphorylated ERK1/2 (pERK1/2, p42/p44) antibody (New England Biolabs, Beverly, MA). The primary antibody was detected using horseradish peroxidase-conjugated goat anti-antibody IgG and visualized by enhanced chemiluminescence Western blotting reagents and Hyperfilm ECL (Amersham Life Sciences International, Buckinghamshire, UK). 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 protein. Proteins were separated by 12% SDS-PAGE, transferred to a polyvinylidene difluoride microporous membrane. Subsequently, membranes were blotted with rabbit anti-phosphorylated ERK1/2 (pERK1/2, p42/p44) antibody (New England Biolabs, Beverly, MA). The primary antibody was detected using horseradish peroxidase-conjugated goat anti-antibody IgG and visualized by enhanced chemiluminescence Western blotting reagents and Hyperfilm ECL (Amersham Life Sciences International, Buckinghamshire, UK). According to the manufacturer’s handbook, Hyperfilm ECL exhibits a linear response to the light produced from enhanced chemiluminescence.
up-take (Fig. 2B). U-0126 together with EGF further inhibited PAH uptake. These data indicate that the MAPK pathway is involved in the normal maintenance of initial basolateral PAH uptake and in the additional stimulation of PAH uptake by EGF.

Phosphorylation of ERK1/2. Because inhibition of MEK reduced the control rate of PAH uptake and eliminated the stimulatory effect of EGF on PAH uptake, we explored the involvement of the MAPK pathway further by determining the degree of phosphorylation of ERK1/2, the step after MEK in the signal cascade, by Western blot analysis in these individual renal tubules. Tubules were incubated as described in Fig. 2A. Quantitative analysis from three independent blots is shown in Fig. 2A. EGF alone tremendously increased ERK activity up to 650% of control. U-0126 leads to an inhibition of ERK activity to 66 ± 10%, whereas, in the presence of EGF, U-0126 diminishes ERK activity to 43 ± 19%. Both results were significantly different from control but not from each other. In direction, these data reflect the decrease of PAH uptake presented above. Although these data fit in direction, we have no mechanistic explanation for the decreases in ERK activation by EGF + U-0126 compared with U-0126 alone. Nevertheless, these data further support the involvement of the MAPK pathway in rabbit renal tubular transport of PAH and its regulation by EGF.

Effect of PGE2 on initial rate of PAH uptake. Because our previous studies with OK cells in culture indicated that regulation of basolateral OAT by EGF via the MAPK pathway continued through the action of PGE2 via binding to a prostanooid receptor (EP4) and subsequent activation of adenylate cyclase and PKA (39, 41), we examined the effect of PGE2 on the initial rate of basolateral PAH uptake in the absence and presence of the PKA inhibitor H89. The preincubation protocol, except for the time periods, was essentially the same as that used with EGF and U-0126. The tubules were preincubated in 1) control Ringer solution for 5 min, 2) control Ringer solution containing 2 μM PGE2 for 5 min, 3) control Ringer solution containing 1 μM H89 for 6 min, and 4) control Ringer solution containing 1 μM H89 for 1 min followed by control Ringer solution containing 1 μM H89 and 2 μM PGE2 for 5 min. Preincubation with PGE2 significantly stimulated PAH uptake, and this stimulation was eliminated by H89 (Fig. 3A).

To further examine whether the EGF stimulation of PAH uptake was mediated via PGE2 and, eventually, PKA, we also examined directly the effect of H89 on EGF stimulation. The preincubation protocol, except for the duration, was again essentially the same as that used above. The tubules were preincubated in 1) control Ringer solution alone for 10 min, 2) control Ringer solution containing 10 ng/ml EGF for 10 min, 3) control Ringer solution containing 1 μM H89 for 11 min, and 4) control Ringer solution containing 1 μM H89 for 1 min.

Fig. 1. Effect of epidermal growth factor (EGF) on initial rate of p-aminohippurate (PAH) uptake in isolated S2 segments of rabbit proximal tubules. Values are means ± SE (n = 5 experiments) expressed as percentage of control, which was set at 100%. Mean control rate of uptake was 1,805.1 ± 136.7 fmol·min⁻¹·mm⁻². *Significantly different from control (P < 0.05).

Fig. 2. Effect of EGF on ERK1/2 activation and PAH uptake in isolated S2 segments of rabbit proximal tubules. A: Western blot results showing effect of EGF (10 ng/ml) and/or U-0126 (10 μM) on phosphorylation of ERK1/2. Antibody resulted in specific staining of protein bands at 42 and 44 kDa, representing phosphorylated ERK1 and ERK2 (pERK1 and pERK2). No other protein signals were detected. Segments were incubated as described in RESULTS (see Effects of inhibition of MEK on initial rate of PAH uptake in the presence and absence of EGF). Values are means from 3 independent blots representing phosphorylated ERK1 and ERK2 (pERK1 and pERK2). No other protein signals were detected. Segments were incubated as described in RESULTS (see Effects of inhibition of MEK on initial rate of PAH uptake in the presence and absence of EGF). Values are means from 3 independent blots. *Significantly different from control (P < 0.05). B: effect of U-0126 and EGF on initial rate of PAH uptake. Values are means ± SE (n = 12 experiments) expressed as percentage of control, which was set at 100%. Mean control rate of uptake was 2,465.5 ± 196.3 fmol·min⁻¹·mm⁻². *Significantly different from control and from each other (P < 0.05).
followed by control Ringer solution containing 1 μM H89 and 10 ng/ml EGF for 10 min. EGF significantly stimulated PAH uptake as expected, and exposure to H89 eliminated this stimulation (Fig. 3B). These data further support the concept that the stimulatory effect of EGF proceeds via PKA.

**Experiments With hOAT1 Expressed in IHKE Cells**

**Stable expression of hOAT1 in IHKE cells.** IHKE cells were transfected with hOAT1 as described in MATERIALS AND METHODS. Five clones were selected by FL-uptake screening after limited-dilution cloning. The two clones showing the highest FL uptake were investigated in more detail. A 606-bp fragment was detected in both clones (IHKE-D9 and IHKE-F10) by RT-PCR with the use of primers against hOAT1 (Fig. 4A).

**Kinetic characterization of IHKE-F10.** Because of the high FL uptake of the IHKE-F10 clone, we decided to use it for our additional studies. For this purpose, we first characterized this clone kinetically. FL uptake by the hOAT1-expressing IHKE cells was concentration dependent (Fig. 5). From these data, we calculated the following kinetic constants: $K_m = 1.5 \mu M$ and $V_{max} = 12 \text{ pmol-mg}^{-1} \cdot \text{min}^{-1}$. This affinity for FL ($K_m$) is in good agreement with published data (56), supporting our decision to perform all subsequent experiments with the IHKE-F10 hOAT1-transfected clone. For all subsequent experiments, 1 μM FL was used.

![Figure 3](http://ajprenal.physiology.org/)

**Fig. 3.** PKA dependence of PGE$_2$ and EGF stimulation of PAH uptake in isolated S2 segments of rabbit proximal tubules. A: effect of PGE$_2$ and H89 on initial rate of PAH uptake. Values are means ± SE ($n = 8$ experiments) expressed as percentage of control, which was set at 100%. Mean control uptake was $2,084.9 ± 236.6 \text{ fmol-min}^{-1} \cdot \text{mm}^{-2}$. *Significantly different from control ($P < 0.05$). B: effect of EGF and H89 on initial rate of PAH uptake. Values are means ± SE ($n = 7$ experiments) expressed as percentage of control, which was set at 100%. Mean control uptake was $1,812.7 ± 206 \text{ fmol-min}^{-1} \cdot \text{mm}^{-2}$. *Significantly different from control ($P < 0.05$).

![Figure 4](http://ajprenal.physiology.org/)

**Fig. 4.** Molecular and functional evidence for expression of human organic anion transporter (hOAT1) in a human kidney epithelial cell line (IHKE). A: detection of a 606-bp fragment specific for hOAT1 in clones transfected with hOAT1 (IHKE-F10 and IHKE-D9) by RT-PCR. Positive control β-actin is present in all cells tested. hOAT1 mRNA was not detected in IHKE wild-type cells. Lanes 1 and 2, IHKE wild type; lanes 3 and 4, IHKE-D9; lanes 5 and 6, IHKE-F10; M, molecular weight marker. B: basolateral uptake of 3 HFLUOROGEN into IHKE wild-type cells and IHKE cells stably expressing hOAT1 (IHKE-F10 and IHKE-D9). No accumulation of fluorescein is seen in wild-type IHKE cells, whereas both hOAT1-expressing clones accumulate fluorescein, indicating active transport. In the presence of 10 mM PAH, fluorescein uptake curves of both hOAT1-expressing IHKE cells resembled IHKE wild-type uptake curve of fluorescein alone.
Phosphorylation of ERK1/2 by EGF. To determine whether the IHKE-hOAT1 cells were a good model system for studying the effects of EGF on OAT, we first examined the effect of EGF on phosphorylation of ERK1/2 by pERK1/2 ELISA. EGF clearly (10 min, 10 ng/ml) led to increased phosphorylation of ERK1/2 in IHKE-hOAT1 cells (Fig. 6A). ERK phosphorylation was reduced in cells treated with U-0126 alone. Treatment with EGF in the presence of U-0126 led to a signal similar to that of U-0126 alone. Thus the ERK1/2 pathway is present in IHKE-hOAT1 cells, and this pathway is activated by 10 min of exposure to EGF. Again, the stimulating effect of EGF on ERK1/2 phosphorylation was inhibited by U-0126. Therefore, it seemed reasonable to investigate the effect of EGF on basolateral organic anion uptake in this cell system expressing a molecularly defined hOAT1.

Effects of inhibition of MEK on initial rate of FL uptake in the presence and absence of EGF. EGF (10 min, 10 ng/ml) increased basolateral FL uptake by ~40% (Fig. 6B). This is about the same amount of EGF-mediated stimulation measured in OK cells and in S2 rabbit proximal tubules (see above and Ref. 39). EGF had no effect on FL uptake in IHKE wild-type cells (data not shown). The observed stimulatory effect of EGF was completely abolished by the MEK inhibitor U-0126 (2.5 μM), whereas U-0126 alone had no effect on basolateral FL uptake (Fig. 6B). The preincubation procedure was identical to that used for the rabbit tubule segments. However, U-0126 inhibits ERK activation, whereas uptake of FL is not affected. Our explanation for this discrepancy is as follows: ERK activity and OAT1 activity do not necessarily behave in a linear fashion. If ERK activity under control conditions is already minimal with respect to its effect on OAT1 activity, additional reduction of ERK activity does not reduce OAT1 activity and, therefore, does not reduce FL uptake further. These data indicate that hOAT1-mediated uptake of organic anions in IHKE-hOAT1 cells is stimulated by EGF via activation of ERK1/2.

Effects of H89 on PGE2 and EGF stimulation. Because PGE2 acting via PKA stimulated organic anion uptake in isolated proximal tubules (Fig. 3A), we investigated whether this could also be the case for the human OAT1 expressed in a human proximal tubule cell line. PGE2 stimulated hOAT1-mediated FL uptake in IHKE-hOAT1 cells (Fig. 7A). This stimulation was completely abolished by inhibition of PKA (by H89). In IHKE wild-type cells, PGE2 exerted no detectable effect on the minimal FL uptake (data not shown).

To examine further whether the EGF action in IHKE-hOAT1 cells was mediated via PGE2 and, eventually, PKA, we determined the effect of H89 on EGF-mediated stimulation of basolateral organic anion uptake. EGF stimulated hOAT1-mediated FL uptake in IHKE-hOAT1 cells (Fig. 7B). This stimulation again was completely abolished by inhibition of PKA with H89. These data clearly indicate that the concept of EGF-stimulated basolateral organic anion uptake proceeding via PGE2 release and subsequent PKA activation holds not...
**DISCUSSION**

In recent studies (39, 40), we investigated basolateral organic anion uptake in the OK cell line. In these studies, we developed a concept for the stimulatory action of EGF on basolateral organic anion uptake that included ERK1/2 activation, arachidonic acid release, and conversion of arachidonic acid to PGE2 via the COX-1 pathway. PGE2 then stimulates organic anion uptake in an autocrine fashion via PKA activation. In the present study, we investigated whether this concept holds for the isolated S2 segment of rabbit renal proximal tubules and for a human renal epithelial cell line stably transfected with the human OAT1.

Because EGF and PGE2 are strongly vasoactive, their effect on renal OAT cannot readily be investigated in whole animal studies. By using isolated proximal tubule segments to investigate the effects of such vasoactive substances on OAT in an intact renal tubule epithelium, systemic influences are avoided. Thus this setup represents a reasonable approach to address whether basolateral organic anion uptake in vivo (or at least in the natural intact renal tubule epithelium) is regulated in a fashion similar to that observed in OK cells. Furthermore, when regulatory processes in particular are considered, it is important to study them with the transporter in its natural cellular environment. Differences in the local environment in immortalized cells may markedly alter regulation, even for the native transporter, in those tissues. This is certainly true also for a heterologous cell system expressing a transfected transporter. Therefore, the present study was undertaken to explore this regulatory concept in an intact renal tubule epithelium and to compare it with the regulatory pattern observed previously in OK cells and with that in a heterologous cell system expressing a transfected transporter.

Recently, OAT1 and OAT3 have been shown to function as the organic anion/α-KG exchanger in renal tubules, at least for rats, mice, and rabbits (49, 50; unpublished observations). It is known that a homolog fragment to OAT1 is present in OK cells showing 90% sequence homology to human OAT1 (7). Organic anion uptake in these cells is kinetically similar to OAT1-mediated transport (42), and there is no evidence that OAT3 exists in this immortalized cell line. Moreover, OAT1 has a high affinity for PAH, whereas OAT3 has only a modest affinity for PAH (humans and rats) (9, 32) or essentially no affinity for PAH (rabbits) (unpublished observations). Because PAH uptake was used in the previous studies with OK cells (39, 40) and in the present studies with S2 segments from rabbit proximal tubules, we can assume that transport involved OAT1. Of course, the IHKE cell line had only been transfected with human OAT1. As indicated in Fig. 4, IHKE wild-type cells show almost no organic anion uptake. Additionally, no OAT1 encoding mRNA is detectable that reflects the kinetic data. Moreover, EGF does not induce any FL uptake in IHKE wild-type cells (data not shown). Thus we are sure that stimulation of organic anion uptake in IHKE-hOAT1 cells is due to stimulation of hOAT1. Therefore, we can assume that these regulatory studies involved effects on OAT1. Whether this pathway can also be involved in regulation of OAT3 is unknown.

In the present study, the effect of EGF on the initial rate of PAH uptake in isolated S2 segments of rabbit renal proximal tubules was virtually identical to that observed previously in OK cells (39, 40). Also, the inhibition of MEK with U-0126 had an effect on PAH uptake and the phosphorylation of ERK1/2 very similar to that observed previously with OK cells (39, 40). As in the OK cells (39), inhibition of MEK reduced control PAH uptake and control phosphorylation of ERK1/2, suggesting that the MAPK pathway is involved in setting the basal level of OAT, probably by OAT1, in intact tubules. Moreover, inhibition of MEK completely blocked the stimu-
EGF and PGE2 Stimulate Organic Anion Uptake by OAT1

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The regulatory effect of EGF on PAH uptake and on ERK1/2 phosphorylation, further implicating the MAPK pathway in the stimulatory effect of EGF on OAT in intact tubules. In addition, the results with isolated tubules supported the previous data with OK cells suggesting that EGF stimulation of OAT involved a continuation of the MAPK pathway, at least through PGE2 and activation of PKA (40). Exposure to PGE2 mimicked exposure to EGF, and the PKA inhibitor H89 blocked stimulation of PAH transport by PGE2 or EGF (Fig. 3, A and B). However, although preincubation of the tubules with H89 alone or with H89 + EGF had no effect on the control rate of PAH uptake (Fig. 3, A and B), preincubation with H89 + PGE2 reduced the rate of PAH uptake significantly below the control level (Fig. 3A). This was a consistent finding in all experiments, but the mechanism involved in the additional inhibitory effect of H89 when used together with PGE2 is not clear.

We stably expressed hOAT1 in a human renal epithelial cell line (IHKE). Expression was verified by RT-PCR experiments and by kinetic characterization (Figs. 4 and 5). In accordance with what was shown for OK cells and rabbit S2 segments, EGF activated ERK1/2 in IHKE-hOAT1 cells. Additionally, EGF stimulated hOAT1-mediated organic anion uptake. Moreover, inhibition of MEK completely blocked the stimulatory effect of EGF on basolateral FL uptake and on ERK1/2 phosphorylation, implicating the MAPK pathway in the stimulatory effect of EGF on hOAT1-mediated organic anion uptake. Inhibition of PKA by H89 had no effect on the control level of hOAT1-mediated FL uptake in IHKE cells (Fig. 7).

In summary, we have obtained additional evidence for the concept of EGF stimulation of organic anion uptake by ERK activation with subsequent PGE2 release and PKA activation in the present study. Isolated rabbit S2 segments and human kidney epithelial cells expressing hOAT1 (IHKE-hOAT1), EGF stimulated organic anion uptake via activation of ERK and PKA. In addition, the presumed intermediate PGE2 stimulated organic anion uptake via PKA in both systems. Because this stimulation occurs in the intact S2 segments of rabbit proximal tubules isolated without enzymatic agents from fresh renal tissue and because it works for the human transporter expressed in a human cell line, it appears very likely that the regulatory cascade EGF-ERK-PLA2-COX1-PGE2-PKA functions in vivo and in humans. Therefore, these observations answer the questions posed in the introduction. Moreover, these stimulatory effects definitively involve at least OAT1. Although consensus sites for PKA phosphorylation are present in OAT1 (6), it cannot be assumed that this regulation occurs through direct phosphorylation of the transporter. Indeed, in view of the data indicating that downregulation of murine OAT1 through PKC does not occur via direct phosphorylation of the transporter, despite many PKC consensus sites (60), we hypothesize that PKA will not act directly on OAT1 but will act via additional intermediates.

Because the OAT 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 pathophysiological 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. For example, some case reports describe a loss of effectiveness of the diuretics acetazolamide or furosemide in patients under long-term COX inhibition therapy (3, 48). Because both diuretics reach the tubular lumen via the proximal tubule organic anion secretion system, their loss of function could be explained by a decrease in basolateral organic anion uptake produced by the COX inhibitors. With respect to EGF, Corrigan et al. (14) described a decreased renal PAH extraction after postischemic acute renal failure in humans leading to severe underestimation of renal blood flow. After renal injury, a rapid fall in EGF mRNA in the kidney was measured (33). We have now shown that the basolateral uptake step of organic anion secretory transport is under stimulatory control of EGF, which is in agreement with excretion data from rats (22). Therefore, a decrease in EGF and, thus, in PAH secretion could explain, at least in part, the data of Corrigan et al.

In addition, PGE2 is considered to be the major renal COX metabolite of arachidonic acid (4). It is known to be produced in the renal cortex (30) and, in particular, in proximal tubule cells (1). Thus the observed mechanism of activation is likely to take place in vivo as well. Inasmuch as the basolateral uptake is considered to be the rate-limiting step in organic anion secretion, we expect that PGE2 induces stimulation of organic anion secretion into the proximal tubule in vivo. PGE2 plays a crucial role in inflammation (53). Its receptors have been detected in the proximal tubule (5, 29). In inflammatory disease of the kidney, PGE2 and its receptors are shown to be upregulated (26). In proximal tubule cells from pigs and humans, PGE2 is generated by reactive oxygen species (27, 54) induced by stress and may directly contribute to renal lesions and loss of kidney function. Inasmuch as basolateral uptake is the rate-limiting step of organic anion secretion, we expect that PGE2 induces stimulation of organic anion secretion into the proximal tubular lumen. Inasmuch as PGE2 is an organic anion itself, this mechanism may reduce the concentration of PGE2 in the kidney interstitium that contradicts its inflammatory potency. We hypothesize that this may represent a mechanism to limit PGE2-induced inflammatory events in the kidney cortex interstitium. Therefore, we further hypothesize that inhibition of organic anion secretion during kidney inflammation may contribute to an increase in severity of inflammation. This will have to be investigated in further experiments.

GRANTS

This work was supported in part by National Institutes of Health Grants DK-56224, ES-0492, and ES-06694 and Deutsche Forschungsgemeinschaft Grant Ge 905/3-4.

REFERENCES


