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Original Article

Regulation of human organic anion transporter 4 by parathyroid hormone-related protein and protein kinase A

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Abstract: Human organic anion transporter 4 (hOAT4) belongs to a family of organic anion transporters that play critical roles in the body disposition of clinically important drugs, including anti-human immunodeficiency virus therapeutics, anti-tumor drugs, antibiotics, antihypertensives, and anti-inflammatories. hOAT4 is abundantly expressed in the kidney and placenta. In the current study, we examined the regulation of hOAT4 by parathyroid hormone-related protein (PTHrP) and protein kinase A (PKA) in kidney COS-7 cells. PTHrP induced a time- and concentration-dependent stimulation of hOAT4 transport activity. The stimulation of hOAT4 activity by PTHrP mainly resulted from an increased cell surface expression without a change in total cell expression of the transporter. Activation of PKA by Bt2-cAMP also resulted in a stimulation of hOAT4 activity through an increased cell surface expression of the transporter. However, PTHrP-induced stimulation of hOAT4 activity could not be prevented by treating hOAT4-expressing cells with the PKA inhibitor H89. We concluded that both PTHrP and activation of PKA stimulate hOAT4 activity through redistribution of the transporter from intracellular compartments to the cell surface. However, PTHrP regulates hOAT4 activity by mechanisms independent of PKA pathway.

Keywords: Drug transporter, parathyroid hormone-related protein, protein kinase A, regulation

Introduction

Human organic anion transporter 4 (hOAT4) belongs to a family of organic anion transporters of 10 members (OAT1-10), which play critical roles in the body disposition of clinically important drugs, including anti-human immunodeficiency virus therapeutics, anti-tumor drugs, antibiotics, antihypertensives, and anti-inflammatories [1-5]. hOAT4 is abundantly expressed in the kidney and placenta [6]. In the kidney, hOAT4 localizes at the apical membrane of the proximal tubule, and functions as an organic anion exchanger for exchanging organic anions across the apical membrane. In the placenta, hOAT4 is localized to the basolateral membrane of syncytiotrophoblasts [7]. It is believed that estrogen biosynthesis in the placenta uses dehydroepiandrosterone sulfate (DHEAS), a precursor produced in large amount by the fetal adrenals. Accumulation of excess DHEAS is associated with intrauterine growth retardation [8]. DHEAS is an OAT4 substrate. Therefore, OAT4 may play an important role in efficient uptake of DHEAS by the placenta for the production of estrogens and for the protection of fetus from the cytotoxicity of DHEAS.

Given such an important role, understanding the regulation of hOAT4 has profound clinical significance. We previously showed that hOAT4 undergoes constitutive internalization from and recycling back to cell surface and that activation of PKC inhibits hOAT4 transport activity through accelerating its internalization rate [9]. hOAT4 transport activity could also be inhibited by pregnancy-specific hormone progesterone (P4). However, P4 regulates hOAT4 activity by mechanisms independent of PKC pathway [10].

Parathyroid hormone-related protein (PTHrP) has been shown to regulate several kidney transporters [11, 12]. The N-terminus of PTHrP is highly homologous to parathyroid hormone

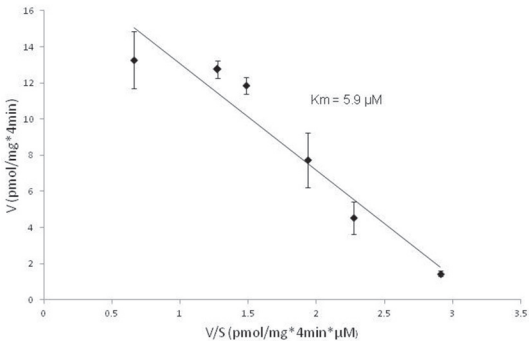

Figure 1. Characterization of hOAT4 in COS-7 cells. Kinetic characteristics were determined at substrate concentration ranging from 0.05 to 30μM (4min uptake). The data represent uptake into hOAT4-expressing cells minus uptake into mock cells. Values are mean ± S.D. (*n* = 3). Transport kinetic values were calculated using the Eadie–Hofstee transformation.

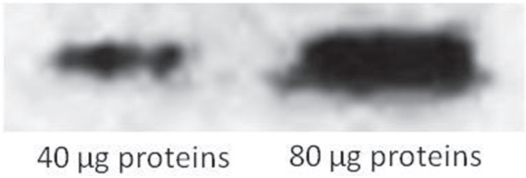

Figure 2. Immunoblot analysis of PTH receptor expression. 40μg and 80μg proteins from COS-7 cell lysates were analyzed by immunoblotting with PTH receptor 1 antibody (1:500).

(PTH), which accounts for its ability to activate PTH/PTHrP receptor in the kidney. In the current study, we investigated the effect of PTHrP on hOAT4 expression and function in kidney COS-7 cells.

Materials and methods

Materials – [^{3}H] estrone sulfate was purchased from Perkin-Elmer Life and Analytical Sciences (Boston, MA, USA). NHS-SS-biotin and streptavidin-agarose beads were purchased from Pierce Chemical (Rockford, IL, USA). Mouse anti-myc antibody was purchased from Roche (Indianapolis, IN, USA). PTHrP fragment (1-34) and all other reagents were from Sigma-Aldrich (St. Louis, MO, USA).

Generation of COS-7 Cells Stably Expressing hOAT4 – Parental COS-7 cells (from African green monkey kidney) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin/streptomycin (100U/ml), and glucose (100mg/ml) in a 5% CO_2 atmosphere at 37°C. Cells were seeded at 5 x 10^5/well of 6-well cluster plate 24h before transfection. cDNA plasmid encoding hOAT4-tagged with myc at its carboxyl terminus was transfected into COS-7 cells using Lipofectamine 2000 reagent, following the manufacturer's instruction. Epitope myc was tagged to hOAT4 to facilitate the immunodetection of hOAT4. After 7 to 8 days of selection in medium containing 0.5mg/ml geneticin (G418; Invitrogen, Carlsbad, CA, USA), resistant colonies were replated into 96 wells for cloning, expansion, and analyzing positive clones. Cells stably expressing hOAT4 were maintained in DMEM medium supplemented with 0.2mg/ml G418, 10% fetal bovine serum, penicillin/streptomycin (100U/ml), and glucose (100mg/ml).

Transport measurement – Cells were plated in 48-well plates. For each well, uptake solution was added. The uptake solution consisted of phosphate-buffered saline/Ca^{2+}/Mg^{2+} (137mM NaCl, 2.7mM KCl, 4.3mM Na_2HPO_4, 1.4mM KH_2PO_4, 1mM $CaCl_2$, and 1mM $MgCl_2$, pH 7.4) and [^{3}H] estrone sulfate. At the times indicated, the uptake was stopped by aspirating off the uptake solution and rapidly washing the well with ice-cold PBS. The cells were then solubilized in 0.2 N NaOH, neutralized in 0.2 N HCl, and aliquotted for liquid scintillation counting. The uptake count was standardized by the amount of protein in each well. Values are means ± S.D. (*n* = 3).

Cell surface biotinylation – Cell surface expression levels of hOAT4 were examined using the membrane-impermeant biotinylation reagent NHS-SS-biotin. The cells were seeded onto six-well plates. After 24 hrs, the medium was removed and the cells were washed twice with 3ml of ice-cold PBS, pH 8.0. The plates were kept on ice, and all solutions were kept ice-cold for the rest of the procedure. Each well of cells was incubated with 1ml of freshly made NHS-SS-biotin (0.5mg/ml in PBS, pH 8.0) in two successive 20min incubations on ice with very gentle shaking. Biotinylation was quenched by first briefly washing each well with 3ml of 100mM glycine and followed by incubation with 100mM glycine on ice for 20min. The cells were then dissolved on ice for 40min in 400μl of lysis buffer [10mM Tris, 150mM NaCl, 1mM EDTA, 0.1% SDS, 1% Triton X-100, and protease inhibitors (200μg/ml phenylmethylsulfonyl fluo-

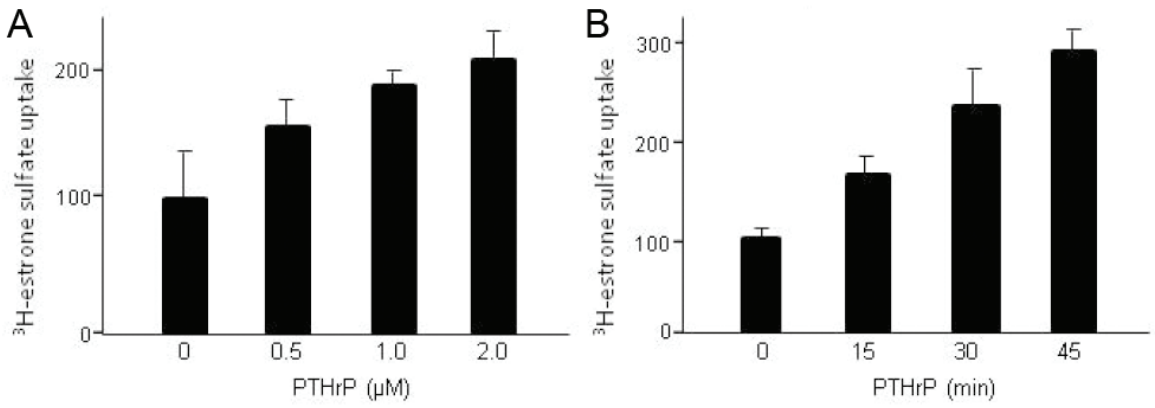

Figure 3. Effect of PTHrP on hOAT4 transport activity. hOAT4-expressing cells were treated with PTHrP at 0-2μM (a), or at 0-45min (b), followed by [^{3}H] estrone sulfate uptake (4min, 100nM). Uptake activity was expressed as a percentage of the uptake measured in untreated cells. The results represent data from 3 experiments. The uptake values in mock cells (parental COS-7 cells) were subtracted. Values are means ± SE (n = 3).

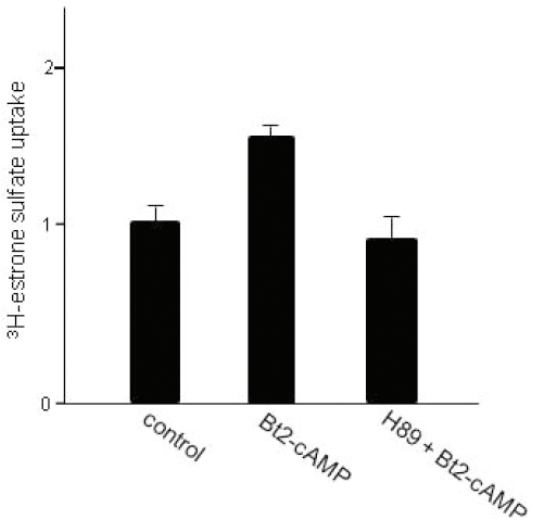

Figure 4. Effect of PKA activator Bt2-cAMP on hOAT4 transport activity. hOAT4-expressing cells were treated with Bt2-cAMP (75μM, 30min) in the presence and absence of PKA inhibitor H89 (10μM, 5min), followed by [^{3}H] estrone sulfate uptake (4min,100nM). Uptake activity was expressed as a percentage of the uptake measured in untreated cells. The results represent data from 3 experiments. The uptake values in mock cells (parental COS-7 cells) were subtracted. Values are means ± SE (n = 3).

ride and 3μg/ml leupeptin), pH 7.4]. The unlysed cells were removed by centrifugation at 16,000 X g at 4°C. Streptavidin-agarose beads were then added to the supernatant to isolate cell membrane protein. hOAT4 was detected in the pool of surface proteins by polyacrylamide gel electrophoresis and immunoblotting using an anti-myc antibody (1:100). Myc was tagged at the carboxyl terminus of hOAT4 for its immunodetection [9].

Electrophoresis and immunoblotting – Protein samples (100μg) were resolved on 7.5% SDS-PAGE mini-gels and electroblotted onto polyvinylidene difluoride membranes. The blots were blocked for 1h with 5% nonfat dry milk in PBS-0.05% Tween, and incubated overnight at 4°C with anti-myc antibody (1:100). The membranes were washed and then incubated with goat anti-mouse IgG conjugated to horseradish peroxidase (1: 5,000), and signals were detected using a SuperSignal West Dura extended duration substrate kit. Images were captured by Fluorchem ®8800 system (Alpha Innotech, San Leandro, CA, USA). Density of bands was analyzed by Quantity One software (Bio-Rad, Hercules, CA, USA).

Data analysis – Each experiment was repeated a minimum of three times. The statistical analysis given was from multiple experiments. Statistical analysis was performed using Student's paired t-tests. A P value < 0.05 was considered significant.

Results

Characterization of hOAT4 in COS-7 Cells – To study the mechanisms underlying regulation of hOAT4-mediated drug transport, we estab-

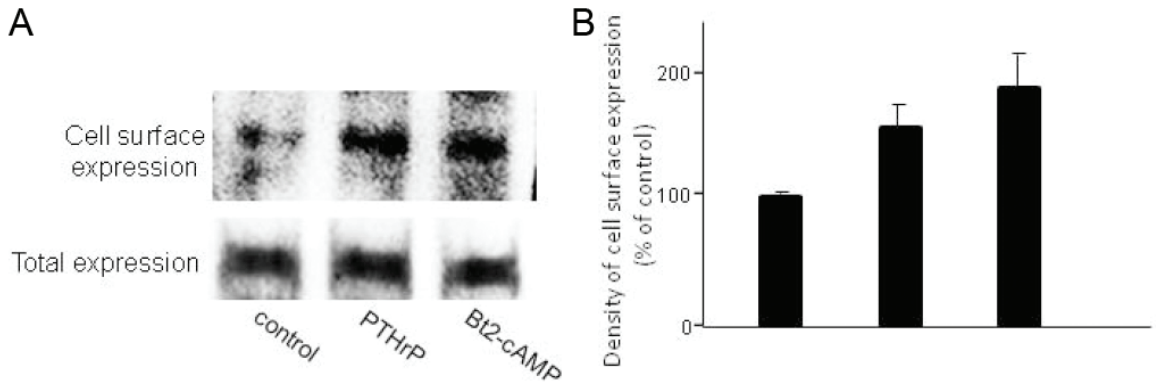

Figure 5. Effects of PTHrP and PKA on cell surface and total cell expression of hOAT4. A: Top panel: Cell surface expression of hOAT4. COS-7 cells stably expressing hOAT4 were treated with PTHrP (2μM, 30min) or PKA activator Bt2-cAMP (75μM, 30min). Treated cells were biotinylated, and the labeled cell surface proteins were precipitated with streptavidin beads and separated by SDS-PAGE, followed by immunoblotting with anti-myc antibody (1:500). Bottom panel: Total expression of hOAT4. COS-7 cells stably expressing hOAT4 were treated with PTHrP or PKA activator Bt2-cAMP. Treated cells were lyzed, followed by immunoblotting with anti-myc antibody. B: Densitometry plot of results from Figure 5a as well as from other experiments. Surface hOAT4 in PTHrP- and Bt2-cAMP-treated cells was expressed as % of surface hOAT4 in control cells. Values are mean ± S.E. (*n* = 3).

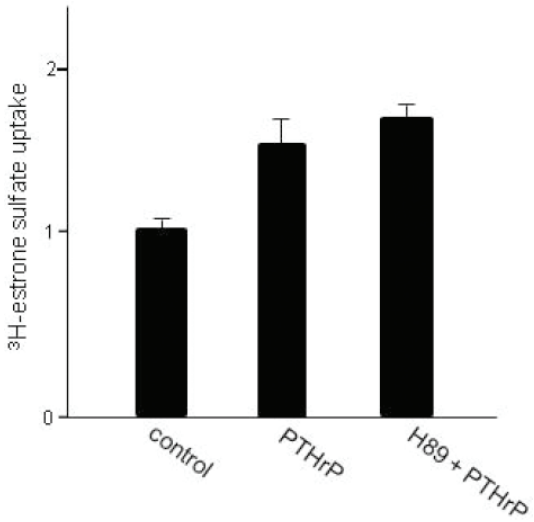

Figure 6. Effect of PKA inhibitor H89 on PTHrP-induced stimulation of hOAT4 activity. hOAT4-expressing cells were pretreated with H89 (10μM, 5min) followed by incubation with PTHrP (2μM, 30min) in the presence or absence of H89. The uptake of [^{3}H] estrone sulfate (4min, 100nM) was then performed. The results represent data from three experiments. The uptake values in mock cells (parental COS-7 cells) were subtracted. Values are mean ± S.D. (*n* = 3).

lished COS-7 cells stably expressing hOAT4. The hOAT4-mediated transport of estrone sulfate across the cell membrane was saturable. Based on Eadie-Hofstee plot analysis (**Figure 1**), the K_m value for estrone sulfate was 5.9μM and V_{max} was 18.9 pmol/mg/4 min. Such transport characteristic is consistent with that of hOAT4 in other system (6), suggesting that COS-7 cells are suitable model for studying hOAT4.

Expression of PTH receptor – PTHrP exerts its action through PTH/PTHrP 1 receptor. We examined whether such receptor is expressed in kidney COS-7 cells. Immunoblotting using anti-PTH receptor antibody detected a specific immuno-reactive band at ~ 80 kDa (**Figure 2**), indicating that COS-7 cells are appropriate system for the investigation of PTHrP action on hOAT4.

Effects of PTHrP on hOAT4 activity – We next examined whether treatment with PTHrP could affect hOAT4 transport activity. Since the hOAT4 expression vector for the current study does not contain the promoter region of hOAT4, the long-term regulation at the transcriptional level cannot be investigated. We only focused on the short-term regulation of the transporter (within a time frame of 1h). PTHrP induced a time- and concentration-dependent stimulation of estrone sulfate uptake (**Figure 3**) with maximum stimulation at 2μM of PTHrP for 30-45min incubation.

Effect of PKA on hOAT4 activity – It was previously shown that PTHrP exerts its effect through several signal transduction pathways including

PKA and PKC [12]. To examine whether PKA has the same effect on hOAT4 as that of PTHrP, we treated cells with PKA activator Bt2-cAMP, followed by measuring hOAT4-mediated uptake of estrone sulfate. As shown in **Figure 4**, Bt2-cAMP significantly stimulated estrone sulfate uptake, which was reversed in the presence of PKA-specific inhibitor H89, suggesting that Bt2-cAMP-stimulated hOAT4 activity is PKA-dependent.

Effect of PTHrP and PKA on hOAT4 expression – To understand the underlying mechanisms of PTHrP- and PKA-stimulated hOAT4 activity, we examined the cell expression of hOAT4. As shown in **Figure 5**, Treatment of hOAT4 expressing cells with PTHrP or Bt2-cAMP resulted in an increase in cell surface expression of hOAT4 without affecting the total expression of the transporter.

Relationship of PTHrP and PKA – Both PTHrP and the PKA activator Bt2-cAMP stimulated hOAT4 activity through an increased cell surface expression of the transporter. This led us to hypothesize that PTHrP exerts its effect through the activation of PKA. To test this hypothesis, we treated hOAT4-expressing cells with PTHrP in the presence of the PKA inhibitor H89. As shown in **Figure 6**, H89 could not reverse the stimulatory effect of PTHrP on hOAT4 activity.

Discussion

The major finding from the current study is that both PTHrP and PKA stimulate hOAT4 transport activity through increasing the surface expression of the transporter. However, PTHrP does not exert its action on hOAT4 through activation of PKA.

To study the regulation of hOAT4, we established COS-7 cells stably expressing the transporter. Although native systems that endogenously express transporters are great assets to identify the endogenous stimuli controlling the transporter function, heterogenous expression systems are useful for asking mechanistic questions with regard to understanding the trafficking and regulation of the transporter. COS-7 cells were directly derived from the kidney and have been very useful in understanding other renal transport processes and cellular functions, including organic cation transport [13, 14]. This cell line does not express endogenous OATs. Therefore, expression of hOAT4 in COS-7 cells will allow us to dissect the transport characteristics of hOAT4 in a relevant mammalian system without the possibly confounding effects of other organic anion transporters. Furthermore, these cells possess endogenous PKC and PKA signaling pathways and provide a good experimental model system for studying the regulatory mechanisms underlying many transport processes [15, 16]. These useful advantages of COS-7 cells make them a powerful system for study of the cloned organic anion transporter. Our functional characterization of hOAT4 (**Figure 1**) showed that the kinetic properties of hOAT4 in these cells are comparable to that of hOAT4 in other systems [9].

Previous studies showed that PTHrP activates PTH/PTHrP receptor in several cell types. In addition, this receptor can couple to multiple G-proteins and thereby activate several signal transduction pathways including PKA and PKC pathways [17-19]. In our study, we showed that both PTHrP and PKA activator Bt2-cAMP stimulated hOAT4 transport activity through increasing the cell surface expression of the transporter. Interestingly, PKA inhibitor H89 could not reverse PTHrP's action, suggesting that PTHrP and PKA exert their action on hOAT4 independently, but not in a synergistic manner. It seems however unlikely that PKC plays a role in this process because we previously showed that activation of PKC led to an inhibition, instead of stimulation, of hOAT4 function [9]. The opposite effects of PKA and PKC on hOAT4 fine-tune its drug transport activity.

An increase in cell surface expression of hOAT4 by treatment with PTHrP or Bt2-cAMP could arise from several possibilities. We recently showed that the members of OAT family undergo constitutive internalization from and recycling back to cell surface [9, 20]. Therefore, an increase in cell surface expression of hOAT4 could result from a decrease in its rate of internalization, an increase in its rate of recycling or a combination of both.

In conclusion, our current study demonstrated that 1) COS-7 cells are suitable model for characterizing hOAT4. 2) Both PTHrP and PKA stimulate hOAT4 transport activity through increasing the surface expression of the transporter. 3) PTHrP regulates hOAT4 activity by mechanisms independent of PKA pathway.

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