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Research Paper

Comparison of the Interaction of Human Organic Anion Transporter hOAT4 with PDZ Proteins between Kidney Cells and Placental Cells

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Purpose. To compare the interaction of human organic anion transporter hOAT4 with PDZ proteins between kidney cells and placental cells.
Materials and Methods. PDZ proteins PDZK1 and NHERF1 were transfected into kidney LLC-PK1 cells and placental BeWo cells expressing hOAT4 or hOAT4-Δ, which lacks the PDZ consensus binding site. The interaction of PDZK1 and NHERF1 with hOAT4 and hOAT4-Δ was investigated by measurement of [^{3}H] estrone sulfate uptake, cell surface and total cell expression of hOAT4.
Results. PDZK1 and NHERF1 enhanced hOAT4 activity in LLC-PK1 cells by increasing the cell surface expression of the transporter. In contrasts, these two PDZ proteins had no effect on hOAT4 activity in BeWo cells.
Conclusion. The interaction of PDZ proteins with hOAT4 may be cell-specific. In placenta, a different set of interacting proteins from PDZK1 and NHERF1 may be required to modulate hOAT4 activity.

KEY WORDS: cell surface biotinylation; co-immunoprecipitation.; drug transporter; estrone sulfate transport; organ-specific interaction; PDZ proteins.

INTRODUCTION

Human organic anion transporter 4 (hOAT4) belongs to a family of organic anion transporters, which play critical roles in the body disposition of clinically important drugs, including anti-HIV therapeutics, anti-tumor drugs, antibiotics, anti-hypertensives, and anti-inflammatories (1–5). The expression of hOAT4 is detected in the kidney and placenta (6). In the kidney, OAT4 functions as an organic anion/dicarboxylate exchanger at the apical membrane of the proximal tubule and is responsible for the reabsorption of organic anions driven by an outwardly directed dicarboxylate gradient (7). In the placenta, hOAT4 is localized to the basolateral membrane of syncytiotrophoblasts (8).

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 (IUGR) (9). 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.

Computer modeling based on hydropathy analysis showed that all the OATs cloned so far contain multiple potential N-linked glycosylation sites in its first extracellular loop. Glycosylation of proteins occurs in two major steps: first step is the addition of oligosaccharides to the nascent protein and the second step is the processing/modification of added oligosaccharides. We previously showed that addition of oligosaccharides but not the processing of the added oligosaccharides plays a critical role in the targeting of hOAT4 to the plasma membrane (10). However, the processing of added oligosaccharides from mannose-rich type to complex type is important for enhancing the binding affinity of hOAT4 for its substrates.

hOAT4 contains a PDZ consensus-binding site (S–T–S–L) at its carboxyl terminus. A relatively large number of PDZ proteins have been identified. These PDZ proteins interact with the PDZ consensus-binding site of its target protein and modulate its function. It has been shown that in human embryo kidney HEK293 cells, PDZ proteins PDZK1 and NHERF1 enhanced hOAT4 function through enhancing the surface expression of the transporter (11). PDZK1 and NHERF1 are also expressed in human placenta (11). However, whether the same set of PDZ proteins interact with hOAT4 in placenta is not known. In the current study, we compared the interaction of these PDZ proteins with hOAT4 in kidney LLC-PK1 cells and human placenta BeWo cells.

MATERIALS AND METHODS

[^{3}H]estrone sulfate was purchased from Perkin-Elmer Life and Analytical Sciences (Boston, MA). NHS-SS-biotin and streptavidin-agarose beads were purchased from Pierce

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Chemical (Rockford, IL). LLC-PK1 cells were purchased from American Type Culture Collection (Manssas, VA). BeWo b30-10 cell line was provided by Dr. Nicholas P. Illsley (Robert Wood Johnson Medical School, University of Medicine & Dentistry of New Jersey, Piscataway, NJ). Human PDZK1 cDNA (Accession Number: BC006518) and human NHERF1 cDNA (accession number, BC011777) are purchased from Open Biosystems (Huntsville, AL). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Construction of epitope-tagged and mutant transporters. To facilitate immunodetection of hOAT4, epitope tag FLAG was added to N-terminus of hOAT4 (hOAT4-N-flag) by site-directed mutagenesis. Other mutant transporters were generated by site-directed mutagenesis using hOAT4 or hOAT4-N-flag as a template. The mutant sequences were confirmed by the dideoxy chain termination method.

Generation of LLC-PK1 cells stably expressing hOAT4 and its mutants. LLC-PK1 cells were grown in Medium 199, supplemented with 10% fetal calf serum, penicillin/streptomycin (100 units/ml) in a 5% CO_2 atmosphere at 37°C. Cells were seeded at 3×10^6/100-mm dish 24 h before transfection. For transfection of transporter cDNA, a Lipofectamine 2000 reagent was used following manufacture's instruction. After 6 days of selection in medium containing 0.8 mg/ml Geneticin (G418; Invitrogen), resistant colonies were replated to 96 wells for cloning, expansion and analyzing positive clones.

Cell culture and transfections. Parental LLC-PK1 cells were grown in Medium 199 containing 10% fetal bovine serum in a 5% CO_2 atmosphere at 37°C. LLC-PK1 cells stable expressing hOAT4, hOAT4-Δ, FLAG tagged hOAT4 and FLAG tagged hOAT4-Δ were grown in Medium with 0.8 mg/ml G418. Parental Bewo b30-10 cells were grown in Dulbecco's modified Eagle's/F-12 medium supplemented with 10% fetal bovine serum, penicillin/streptomycin (100 U/ml), and glucose (100 mg/ml). Bewo b30-10 cells stable expressing hOAT4 were grown in medium containing 0.5 mg/ml G418. Cells were grown to 90–100% confluency and transfected with the appropriate plasmids using Lipofectamine 2000 (Invitrogen).

Transport Measurements. For each well, uptake solution was added. The uptake solution consisted of phosphate-buffered saline (PBS)/CM (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 0.1 mM $CaCl_2$, and 1 mM $MgCl_2$, pH 7.3) and [^{3}H]estrone sulfate (50 nM). At the times indicated in the figure legends, the uptake was stopped by aspirating the uptake solution off and rapidly washing the cells with ice-cold PBS solution. Transport activity was also measured in cell monolayers cultured in transwell chambers (Costar, Cambridge, MA). To prepare cell monolayers, cells were seeded at a density of 1.0×10^5 cells per polycarbonate membrane (0.4 μm pore size, collagen coated) in transwell cell chambers, which were placed in 12-well cluster plates. The volumes of medium inside and outside the chambers were 0.5 and 1.5 ml, respectively. Fresh medium was replaced every day, and the cells were used between the third and fourth days after seeding. To measure the cellular uptake of radiolabeled substrates, the reaction was initiated by adding substrate to the apical side or the basal side of the monolayers. After incubation for a specified period, the uptake medium was aspirated and discarded, and the membrane was rapidly washed three times 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.

Cell surface biotinylation. Cell surface expression levels of hOAT4 and its mutants were examined using the membrane-impermeable biotinylation reagent, NHS-SS-biotin (Pierce). hOAT4 and its mutants were expressed in cells grown in six-well plates using Lipofectamine 2000 as described above. After 24 h, the medium was removed and the cells were washed twice with 3 ml of ice-cold PBS/CM (pH 8.0). The plates were kept on ice, and all solutions were ice-cold for the rest of the procedure. Each well of cells was incubated with 1 ml of NHS-SS-biotin (0.5 mg/ml in PBS/CM) in two successive 20-min incubations on ice with very gentle shaking. The reagent was freshly prepared for each incubation. After biotinylation, each well was briefly rinsed with 3 ml of PBS/CM containing 100 mM glycine and then incubated with the same solution for 20 min on ice to ensure complete quenching of the unreacted NHS-SS-biotin. The cells were then dissolved on ice for 1 h in 400 μl of lysis buffer [10 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100 with 1:100 protease inhibitor mixture (Sigma)]. The cell lysates were cleared by centrifugation at 16,000×*g* at 4°C. Fifty microliters of streptavidin-agarose beads (Pierce) was then added to the supernatant to isolate cell membrane protein. hOAT4 and its mutants were detected in the pool of surface proteins by SDS–PAGE and immunoblotting.

Electrophoresis and immunoblotting. Protein samples were resolved on 7.5% SDS–PAGE minigels and electroblotted on to polyvinylidene difluoride membranes. The blots were blocked for 1 h with 5% nonfat dry milk in PBS, 0.05%

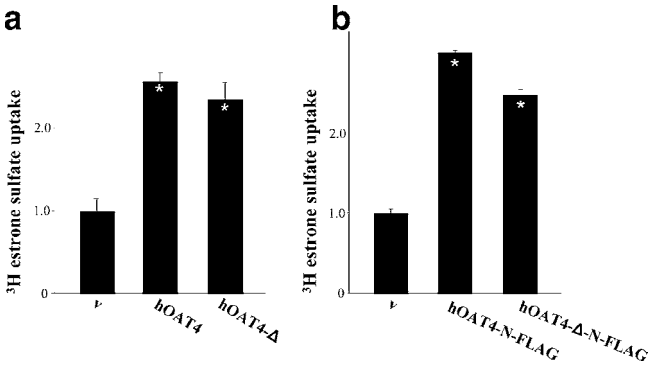

Fig. 1. Estrone sulfate uptake. **a** Estrone sulfate uptake into cells expressing hOAT4 and hOAT4-Δ. [^{3}H]estrone sulfate uptake (4 min, 100 nM) was measured. Uptake activity was expressed as a percentage of the uptake measured in mock cells (*v*). The results represent data from three experiments. Values are mean±SE(*n*=3). *Asterisks* indicate values significantly different ($p<0.05$) from that of mock control. **b** Estrone sulfate uptake into cells expressing FLAG-tagged hOAT4 and hOAT4-Δ. Uptake activity was expressed as a percentage of the uptake measured in mock cells (*v*). The results represent data from three experiments. Values are mean±SE (*n*=3). *Asterisks* indicate values significantly different ($p<0.05$) from that of mock control.

Tween 20, washed, and incubated for 1 h at room temperature with appropriate primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies. The signals were detected by SuperSignal West Dura extended duration substrate kit (Pierce). Nonsaturating, immunoreactive protein bands were quantitated by scanning densitometry with the FluorChem 8000 imaging system (Alpha Innotech Corp., San Leandro, CA).

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 of 0.05 was considered significant.

RESULTS

Transport activity of hOAT4 and hOAT4-Δ. To investigate the interaction of hOAT4 with PDZ proteins, we constructed hOAT4-Δ, which lacks the last three amino acids (PDZ consensus binding site). Measurement of ^{3}H-labeled estrone sulfate transport into LLC-PK1 cells transfected with hOAT4 and hOAT4-Δ showed that both transporter proteins induced significant uptake as compared to that of mock control (Fig. 1a). To facilitate the detection of these transporter proteins, epitope FLAG was tagged to the amino terminus of hOAT4 and hOAT4-Δ so that the epitope would not be expected to interfere with interaction of the carboxyl terminus with PDZ proteins. As shown in Fig. 1b, the pattern of the transport activity FLAG-tagged hOAT4 and hOAT4-Δ mimicked their untagged counter parts, suggesting that the tagged transporters retained the functional properties of their parental transporters.

Interaction of FLAG-tagged hOAT4 and FLAG-tagged hOAT4-Δ with PDZK1 and NHERF1. To facilitate the further characterization of hOAT4 and hOAT4-Δ with PDZ proteins, we established LLC-PK1 cells stably expressing of both transporters. Transfection of PDZ proteins PDZK1 and NHERF1 into LLC-PK1 cells (plated on 12-well plates)

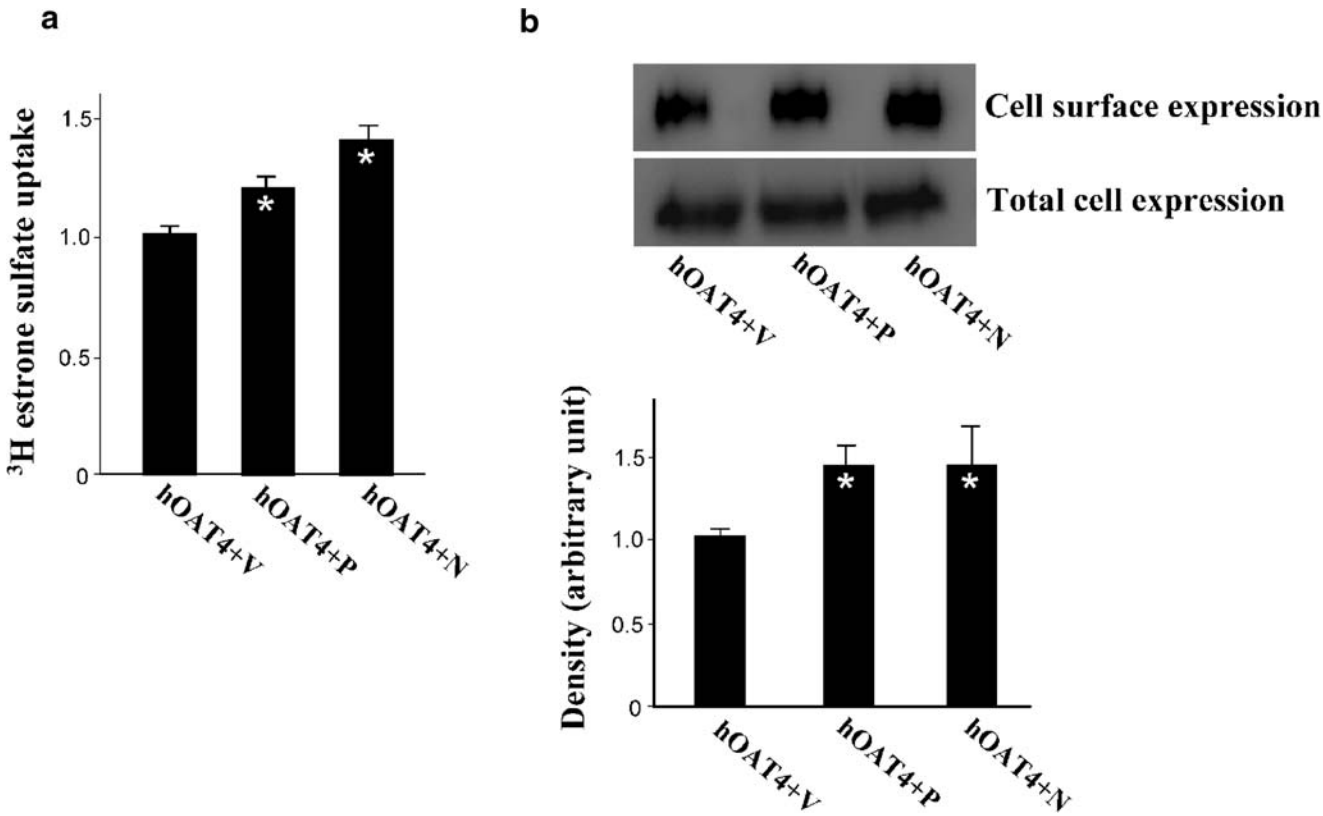

Fig. 2. Effect of PDZK1 and NHERF1 on FLAG-tagged hOAT4 and FLAG-tagged hOAT4-Δ. **a** Functional effect of PDZK1 and NHERF1 on FLAG-tagged hOAT4 in LLC-PK1 cells. FLAG-tagged hOAT4 expressing cells were transiently transfected with PDZK1, NHERF1 or vector followed by [^{3}H]estrone sulfate uptake (4 min, 100 nM). *V* Vector, *P* PDZK1, *N* NHERF1. Uptake activity was expressed as a percentage of the uptake measured in cells expressing FLAG-tagged hOAT4 alone. The results represent data from three experiments. Values are mean±SE (n=3). **b** Effect of PDZK1 and NHERF1 on cell surface and total cell expression of FLAG-tagged hOAT4. *Top panel* For cell surface expression cells stably expressing FLAG-tagged hOAT4 were biotinylated, and the labeled cell surface proteins were precipitated with streptavidin beads, separated by SDS–PAGE, followed by Western blotting with anti-FLAG antibody (1:500). *V* Vector, *P* PDZK1, *N* NHERF1. For total cell expression, cells stably expressing FLAG-tagged hOAT4 were lysed and separated by SDS–PAGE, followed by Western blotting with anti-FLAG antibody (1:500). *V* vector, *P* PDZK1, *N* NHERF1. *Bottom panel* Densitometry analysis of the ratio of the band densities of cell surface expression vs total cell expression. *Asterisks* indicate values significantly different (p<0.05) from that of control (hOAT4 +V).

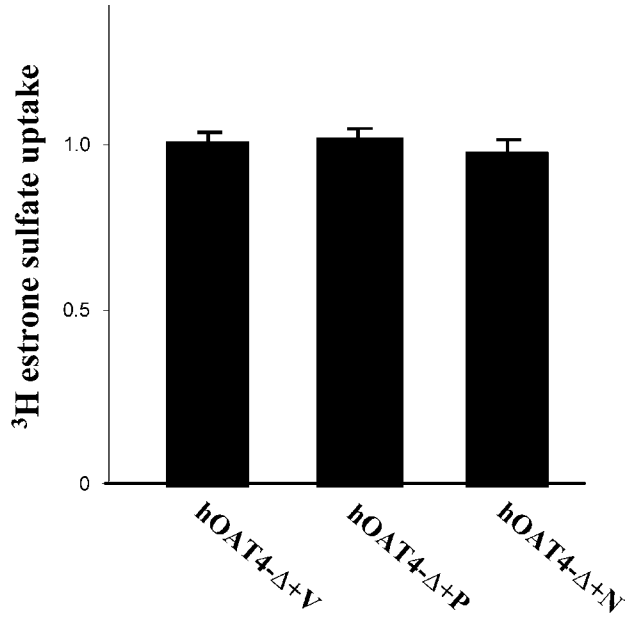

Fig. 3. Functional effect of PDZK1 and NHERF1 on FLAG-tagged hOAT4-Δ in LLC-PK1 cells. hOAT4-Δ expressing cells were transiently transfected with PDZK1, NHERF1 or vector (*v*) followed by [^{3}H]estrone sulfate uptake (4 min, 100 nM). Uptake activity was expressed as a percentage of the uptake measured in cells expressing FLAG-tagged hOAT4-Δ alone. The results represent data from three experiments. Values are mean±SE (n=3).

stably expressing FLAG-tagged hOAT4 significantly increased transport activity of the transporter (Fig. 2a). Such increase in transport activity was accompanied by an increase in cell surface expression of the transporter (Fig. 2b) without a change in its total cell expression (Fig. 2c). In contrast, transfection of PDZ proteins PDZK1 and NHERF1 into LLC-PK1 cells stably expressing FLAG-tagged hOAT4-Δ had no effect on transport activity of the transporter (Fig. 3).

Interaction of FLAG-tagged hOAT4 and FLAG-tagged hOAT4-Δ with PDZK1 and NHERF1 in polarized LLC-PK1 cells. The above experiments were carried out in LLC-PK1 cells plated in 48-well plate. When plated on transwells, LLC-PK1 cells polarized into apical and basolateral membrane domains, mimicking kidney proximal tubule cells *in vivo*. PDZ proteins have been shown to modulate the function of transporters at both the apical and the basolateral membranes (12,13). Estrone sulfate uptake was measured at both the apical and the basolateral membranes. Both the apical and basolateral membranes induced significant amount of uptake in cells expressing FLAG-tagged hOAT4 and FLAG-tagged hOAT4-Δ as compared to that of mock cells (not shown). The estrone sulfate uptake was then measured in polarized cells after the transfection of PDZK1 or NHERF1. As shown in Fig. 4, both PDZK1 and NHERF1 induced an increased transport activity of hOAT4 at the apical side, whereas there

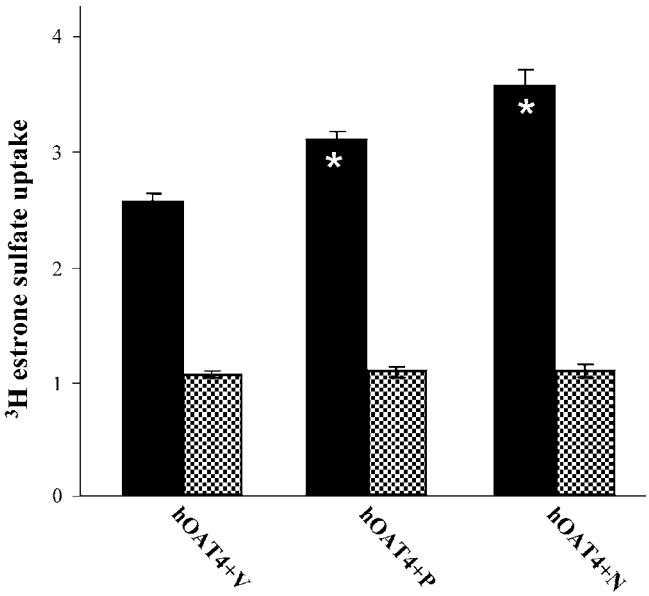

Fig. 4. Interaction of FLAG-tagged hOAT4 and FLAG-tagged hOAT4-Δ with PDZK1 and NHERF1 in polarized LLC-PK1 cells. Cells expressing FLAG-tagged hOAT4 were seeded on transwells followed by transient transfection of PDZK1 or NHERF1. Directional uptake of estrone sulfate (100 nM, 4 min) from either apical sides (*solid columns*) or basal sides (*dotted columns*) were then performed. *V* Vector, *P* PDZK1, *N* NHERF1.Uptake activity was expressed as a percentage of the basal uptake measured in cells expressing FLAG-tagged hOAT4 alone. The results represent data from three experiments. Values are mean±SE (n=3).

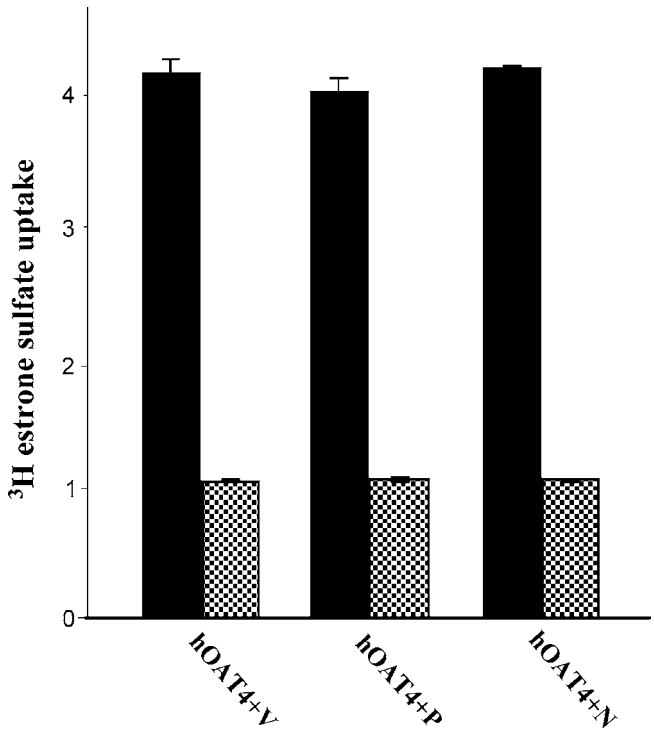

Fig. 5. Interaction of hOAT4 with PDZK1 and NHERF1 in polarized BeWo cells. Cells expressing hOAT4 were seeded on transwells followed by transient transfection of PDZK1 or NHERF1. Directional uptake of estrone sulfate (100 nM, 4 min) from either apical sides (*solid columns*) or basal sides (*dotted columns*) were then performed. *V* Vector, *P* PDZK1, *N* NHERF1. Uptake activity was expressed as a percentage of the basal uptake measured in cells expressing hOAT4 alone. The results represent data from three experiments. Values are mean±SE (n=3).

was no effect of PDZK1 and NHERF1 on hOAT4 activity on the basolateral side. In cells expressing hOAT4-Δ, PDZK1 and NHERF1 had no effect on transport activity at both the apical and the basolateral sides (not shown).

Effect of PDZK1 and NHERF1 on hOAT4 in BeWo cells. In addition to being expressed in the kidney, hOAT4, PDZK1 and NHERF1 are also abundantly expressed in human placenta (11). To explore whether PDZK1 and NHERF1 has any effect on hOAT4 in placenta, we transfected PDZK1 and NHERF1 into human placenta BeWo cells stably expressing hOAT4 followed by measurement of estrone sulfate uptake into either the apical or the basolateral sides of the cells. As shown in Fig. 5, transfection of PDZK1 and NHERF1 had no effect on hOAT4 function on both the apical and the basolateral sides.

DISCUSSION

hOAT4 belongs to a family of organic anion transporters, which play critical roles in the body disposition of clinically important drugs. hOAT4 is abundantly expressed in the kidney and placenta. Although the regulation of hOAT4 in the kidney has begun to be explored, its regulation in the placenta is largely unknown. We have recently shown that hOAT4 activity in placental BeWo cells was down regulated by both activation of protein kinase C and pregnancy-specific hormones progesterone. However, progesterone regulates hOAT4 activity by mechanisms independent of PKC pathway (14). In the current study, we compared the interaction of hOAT4 with two PDZ proteins PDZK1 and NHERF1 in kidney LLC-PK1 cells and human placental BeWo cells.

Miyazaki *et al*. previously showed (11) that PDZK1 and NHERF1 increased hOAT4 activity in HEK293 cells, which was accompanied by an increased cell surface expression of the transporters. However, whether such increase in cell surface expression was caused by an increase in total protein synthesis or was caused by a trafficking of already existent intracellular hOAT4 to the cell surface was not examined. Furthermore, PDZ proteins have been shown to modulate transporter activity at both the apical and the basolateral surface of the transport epithelia. For example, it has been shown that PDZK1 regulated transport activity of an urate/anion exchanger URAT1 on the apical side of the kidney proximal tubule cells (12) and an organic anion transporting protein Oatp1a1 at the basolateral side of liver hepatocytes (13). HEK293 cells are nonpolarized cells and therefore, polarized regulation of hOAT4 by PDZ proteins could not be explored. In the current study, we expanded their previous observation by showing that PDZK1 and NHERF1 only enhanced transport activity of hOAT4 at the apical side but not the basolateral side, and that the increased transport activity was due to an increased cell surface expression without affect the total cell expression of the transporter. Our finding suggests that PDZ proteins affect hOAT4 activity by affecting its trafficking between cell surface and intracellular compartment. PDZK1 and NHERF1 also increased hOAT4 activity in COS-7 cells, another kidney cell line (data not shown). Therefore, modulation of hOAT4 activity by PDZK1 and NHERF1 is probably the feature of kidney cells.

In the current study we also provided interesting data showing that although hOAT4, PDZK1 and NHERF1 are all present in the placenta (11), transfection of PDZK1 and NHERF1 into placental BeWo cells expressing hOAT4 had no effect on the transport activity of the transporter on both the apical and the basolateral sides, which is in contrast to what we observed of the interactions between these proteins in kidney cells LLC-PK1 and COS-7. This finding suggests that interaction of hOAT4 with PDZ proteins may depend on specific organ. It is possible that in placenta, a different set of proteins regulate OAT4 function. However, we can't exclude the possibility that hOAT4 in BeWo cells was already fully engaged in the interaction with endogenously expressed PDZK1, NHERF1 or similar PDZ proteins. Further increase in PDZ protein level will not increase hOAT4 activity.

In conclusion, we showed that PDZ proteins PDZK1 and NHERF1 are regulators of hOAT4 function in kidney cells, whereas the same set of PDZ proteins has no effect on hOAT4 function in placenta cells, suggesting that the interacting partners of hOAT4 in placenta maybe different from that in kidney.

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