Regulation of human organic anion transporter 4 by progesterone and protein kinase C in human placental BeWo cells

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Zhou F, Hong M, You G. Regulation of human organic anion transporter 4 by progesterone and protein kinase C in human placental BeWo cells. Am J Physiol Endocrinol Metab 293: E57–E61, 2007. First published March 6, 2007; doi:10.1152/ajpendo.00696.2006.—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 placenta. In the current study, we examined the regulation of hOAT4 by pregnancy-specific hormones progesterone (P4) and 17β-estradiol (E2) and by protein kinase C (PKC) in human placental BeWo cells. P4 induced a time- and concentration-dependent down-regulation of hOAT4 transport activity, whereas E2 had no effect on hOAT4 function. The downregulation of hOAT4 activity by P4 mainly resulted from a decreased cell surface expression without a change in total cell expression of the transporter, kinetically revealed as a decreased V_{max} without significant change in K_m. Activation of PKC by phorbol 12,13-dibutyrate also resulted in an inhibition of hOAT4 activity through a decreased cell surface expression of the transporter. However, P4-induced downregulation of hOAT4 activity could not be prevented by treating hOAT4-expressing cells with the PKC inhibitor staurosporine. We concluded that both P4 and activation of PKC inhibited hOAT4 activity through redistribution of the transporter from cell surface to the intracellular compartments. However, P4 regulates hOAT4 activity by mechanisms independent of PKC pathway.

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 (3, 7, 18, 19, 24). hOAT4 is abundantly expressed in the kidney and placenta (4). 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 (6). In the placenta, hOAT4 is localized to the basolateral membrane of syncytiotrophoblasts (20). 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 (17). 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 has shown that hOAT4 contains multiple potential glycosylation sites in its first extracellular loop. The glycosylation process occurs in two major steps: the first step is the addition of oligosaccharides to the nascent protein, and the second step is the processing of added oligosaccharides during which the added oligosaccharides are modified and trimmed. Our group (27) 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. Processing of added oligosaccharides may not be essential in determining the substrate spectra of hOAT4 and its property as an organic anion exchanger. 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.

To date, little is known about how hOAT4 is regulated in the placenta. Progesterone (P4) and 17β-estradiol (E2) are the two most important steroid hormones produced by the human placenta during pregnancy. These hormones have been shown to affect the expression and activities of other placental transporters (22). In the present study, we investigated the effects of these hormones on hOAT4 function in human placental BeWo cells and the possible interaction of these hormones with PKC pathway.

MATERIALS AND METHODS

Materials. [3H]estrone sulfate was purchased from Perkin-Elmer Life and Analytical Sciences (Boston, MA). NHS-SS-biotin and streptavidin-agarose beads were purchased from Pierce Chemical (Rockford, IL). P4 (P-8783), E2 (E-2758), staurosporine from Streptomyces species (S-5921), and phorbol 12,13-dibutyrate (PDBu; P-1269) were purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco’s modified Eagle’s/F-12 medium (phenol red free) was purchased from GIBCO (Grand Island, NY). Charcoal/dextran-stripped fetal bovine serum was purchased from HyClone (Logan, UT).

Cell culture. Parental BeWo b30-10 cells were grown in Dulbecco’s modified Eagle’s/F-12 medium (phenol red free) supplemented with 5% charcoal/dextran-striped fetal bovine serum, penicillin/streptomycin (100 U/ml), and glucose (100 mg/ml) in a 5% CO2 atmosphere at 37°C. BeWo b30-10 cells stably expressing hOAT4 (25) were maintained in Dulbecco’s modified Eagle’s/F-12 medium (phenol red free) supplemented with 5% charcoal/dextran-striped fetal bovine serum, 0.5 mg/ml genetin (G418; Invitrogen, Carlsbad, CA), and glucose (100 mg/ml) in a 5% CO2 atmosphere at 37°C.

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Transport measurement. Cells plated in 48-well plates were treated with each reagent at 37°C for certain time periods as indicated. For each well, uptake solution was added. The uptake solution consisted of phosphate-buffered saline/Ca2+/Mg2+ (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.4 mM KH2PO4, 1 mM CaCl2, and 1 mM MgCl2, pH 7.4) and [3H]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 ± SE (n = 3).

Cell surface biotinylation. Cell surface expression levels of hOAT4 were examined using the membrane-impermeant biotinylation reagent NHS-SS-biotin (Pierce Chemical). The BeWo b30-10 cells stably expressing hOAT4 and parental BeWo b30-10 cells were seeded onto six-well plates at 8 × 105 cells per well. After 24 h, the medium was removed and the cells were washed twice with 3 ml 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 1 ml of NHS-SS-biotin (0.5 mg/ml in PBS) in two successive 20-min incubations on ice with very gentle shaking. The reagent was freshly prepared for incubation. After biotinylation, each well was briefly rinsed with 3 ml of PBS 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, and protease inhibitors (200 μg/ml phenylmethylsulfonyl fluoride and 3 μg/ml leupeptin), pH 7.4]. The unlysed cells were removed by centrifugation at 13,000 rpm at 4°C. Streptavidin–agarose beads (50 μl; Pierce Chemical) 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-hOAT4 antibody (Alpha Diagnostic International, San Antonio, TX).

Electrophoresis and Western blotting. Protein samples (100 μg) were resolved on 7.5% SDS-PAGE minigels and electroblotted onto polyvinylidene difluoride membranes. The blots were blocked for 1 h with 5% nonfat dry milk in PBS-0.05% Tween, washed, and incubated overnight at 4°C with polyclonal anti-hOAT4 antibody (1:500; Alpha Diagnostic International). The membranes were washed and then incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (1:5,000), and signals were detected using a SuperSignal West Dura extended duration substrate kit (Pierce Chemical).

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

Effects of P4 and E2 on hOAT4 function. We first examined whether treatment with P4 or E2 could affect hOAT4 transport activity in BeWo cells. 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 1 h). P4 induced a time- and concentration-dependent inhibition of estrone sulfate uptake (Fig. 1A), whereas E2 had no significant effect on hOAT4 function (Fig. 1B). To further examine the mechanism of P4-induced downregulation of hOAT4 activity, we determined [3H]estrone sulfate uptake at different substrate concentrations. An Eadie-Hofstee analysis of the derived data (Fig. 2) shows that pretreatment with P4 resulted in a decreased Vmax (0.154 ± 0.004 pmol·μg−1·min−1 with untreated cells and 0.080 ± 0.025 pmol·μg−1·min−1 in the presence of P4) with no significant change in the affinity for estrone sulfate (14.3 ± 0.6 μM with untreated cells and 13.2 ± 0.5 μM in the presence of P4). Determination of the protein concentrations in control cells confirmed that P4 treatment did not change the total protein content of the cultures (data not shown).

Effect of P4 on hOAT4 expression. A decreased Vmax could be affected by either a reduced number of the transporter at the

Fig. 1. Effect of progesterone (P4) and 17β-estradiol (E2) on human organic anion transporter 4 (hOAT4) activity in BeWo cells. hOAT4-expressing BeWo b30-10 cells were treated with P4 (A) or E2 (B) at various concentrations for 30 min and 1 h, followed by [3H]estrone sulfate uptake (4 min, 100 nM). 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 BeWo b30-10 cells) were subtracted. Values are means ± SE (n = 3).
cell surface or a reduced transporter turnover number (1, 9, 12, 21, 26). To differentiate between these possibilities, we determined transporter expression both at the cell surface and in the total cell lysates. We showed that P4 treatment resulted in a reduced cell surface expression of hOAT4 without affecting the total cell expression of the transporter (Fig. 3).

**Effect of activation of protein kinase C on hOAT4 expression.** Our group previously showed (25) that activation of protein kinase C (PKC) by phorbol 12-myristate 13-acetate and PDBu led to an inhibition of hOAT4 activity. However, the mechanism underlying such inhibition was not investigated in that study. In the current study, we examined whether such inhibition also, like the effect of P4, resulted from a decreased cell surface expression of the transporter. Our results show that, indeed, similar to the effect of P4, PDBu treatment also resulted in a reduced cell surface expression of hOAT4 without affecting its total cell expression (Fig. 4).

**Relationship between P4 and PKC.** Both P4 and the PKC activator PDBu inhibited hOAT4 activity through a decreased cell surface expression of the transporter. This led us to hypothesize that P4 exerts its effect through the activation of PKC. To test this hypothesis, we treated hOAT4-expressing BeWo cells with P4 in the presence of the PKC inhibitor staurosporine. As shown in Fig. 5, although staurosporine efficiently reversed the inhibitory effect of PDBu on hOAT4 activity as well as its cell surface expression, it could not reverse the inhibitory effect of P4 on hOAT4 activity and its cell surface expression.

**DISCUSSION**

hOAT4 belongs to a family of organic anion transporters that 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. The present study investigated the regulation of hOAT4 in human placental BeWo cells by steroid hormones P4 and E2 and by PKC and explored the mechanisms underlying their regulation. We found that P4 acutely inhibited hOAT4...
activity at a relatively high concentration of 10^{-5} M (Fig. 1). It was reported that the plasma P_4 concentration at term is \sim 0.7 \times 10^{-6} M, and the intracellular P_4 concentrations in placenta were \sim 12 times higher than those in maternal plasma (11). Therefore, 10^{-5} M P_4 could be reached in the placenta at term. The plasma E_2 concentrations are around 10^{-8} and 10^{-7} M during pregnancy (2, 11). However, we did not observe any significant effect of E_2 on hOAT4 activity within a concentration range of 10^{-8}–10^{-5} M (Fig. 1).

Our kinetic analysis of the inhibition of hOAT4 activity by P_4 (Fig. 2) showed that the reduced transport activity was contributed by a reduced maximum transport velocity (V_{max}) without affecting the binding affinity (1/K_m) for the substrates. V_{max} can be affected by either the number of the transporter at the cell surface or the transporter turnover number (1, 9, 12, 21, 26). To differentiate between these possibilities, we determined the effect of P_4 on hOAT4 expression both at the cell surface and in the total cell lysates. Our results showed that P_4 treatment resulted in a reduced cell surface expression of hOAT4 without affecting its total cell expression (Fig. 3), suggesting that a redistribution of hOAT4 from cell surface to the intracellular compartments occurred during such treatment. Such redistribution was observed previously from other membrane transporters (10, 13). In response to stimuli, these transporters were removed from the cell surface to intracellular compartments, where they waited for the next signal to recycle back to the cell surface. An example is the Na^++K^+-ATPase (16). Treatment of Xenopus oocytes with progesterone resulted in the retrieval of both endogenously expressed and exogenously injected Na^++,K^+-ATPase from the cell surface. The treatment of progesterone also led to an increased endocytotic activity. Coated pits and vesicles appeared in the oocytes plasma membrane that might be involved in endocytosis, suggesting that progesterone-induced redistribution of Na^++,K^+-ATPase may occur through an endocytotic pathway. Whether such a pathway is also involved in progesterone-induced rapid redistribution of hOAT4 needs further investigation.

Fig. 5. P_4-induced inhibition of hOAT4 activity and surface expression is independent of activation of PKC. A, top: effect of staurosporine on PDBu-induced inhibition of hOAT4 activity. BeWo b30-10 Cells stably expressing hOAT4 were pretreated with staurosporine (2 \mu M, 5 min) followed by incubation with PDBu (10^{-6} M, 15 min) in the presence or absence of staurosporine (2 \mu M). The uptake of [^{3}H]estrone sulfate (4 min, 100 nM) was then performed. Middle: effect of staurosporine on PDBu-induced inhibition of hOAT4 surface expression. BeWo b30-10 Cells stably expressing hOAT4 were pretreated with staurosporine (2 \mu M, 5 min) followed by incubation with PDBu (10^{-6} M, 15 min) in the presence or absence of staurosporine (2 \mu M). Cell surface biotinylation was then performed. Labeled cell surface proteins were precipitated with streptavidin beads, separated by SDS-PAGE, followed by Western blotting with anti-hOAT4 antibody (1:500). Bottom: the intensity of the transporter expression from the experiment shown in middle and other experiments was quantified. Values significantly different (P < 0.05) from that of PDBu-treated cells. B, top: effect of staurosporine on P_4-induced inhibition of hOAT4 activity. hOAT4-expressing cells were pretreated with staurosporine (2 \mu M, 5 min) followed by incubation with P_4 (10^{-5} M, 1 hr) in the presence or absence of staurosporine (2 \mu M). The uptake of [^{3}H]estrone sulfate (4 min, 100 nM) was then performed. The results represent data from three experiments. The uptake values in mock cells (parental BeWo b30-10 cells) were subtracted. Values are mean \pm SE (n = 3). Middle: effect of staurosporine on P_4-induced inhibition of hOAT4 surface expression. BeWo b30-10 Cells stably expressing hOAT4 were pretreated with staurosporine (2 \mu M, 5 min) followed by incubation with P_4 (10^{-5} M, 1 hr) in the presence or absence of staurosporine (2 \mu M). Cell surface biotinylation was then performed. Labeled cell surface proteins were precipitated with streptavidin beads, separated by SDS-PAGE, followed by Western blotting with anti-hOAT4 antibody (1:500). Bottom: the intensity of the transporter expression from the experiment shown in middle and other experiments was quantified. Values significantly different (P < 0.05) from that of P_4-treated cells.
The physiological significance of downregulation of hOAT4 function by P₄ remains speculative. ABCG2, also called breast cancer resistance protein and an efflux pump for various compounds in placenta, was also shown to be downregulated by P₄ in placental BeWo cells (23). Several studies (8, 14, 15) reported a gestational age-dependent decrease in the expression of p-glycoprotein (P-gp), suggesting that placental P-gp expression is under developmental control. Considering the role as an efflux pump for xenobiotics, the gestational age-dependent expression of P-gp in placenta makes teleological sense. The fetus is at greatest danger to toxic insult from xenobiotics early in pregnancy. Therefore, upregulation of the expression of P-gp early in pregnancy is a mechanism used to protect the fetus from toxicological insult. It is known that progesterone concentration increases with gestational age. This led us to hypothesize that hOAT4 expression in placenta may also be developmentally regulated with the highest expression in early pregnancy. We are currently testing such a hypothesis.

In a previous study, our group (25) showed that activation of PKC by PDBu led to an inhibition of hOAT4 activity in BeWo cells. However, the mechanism underlying such inhibition was not investigated. In the present study, we showed that PDBu treatment resulted in a reduced cell surface expression of hOAT4, without affecting its total cell expression (Fig. 4), suggesting that like the effect of P₄, PDBu also caused a redistribution of hOAT4 from cell surface to the intracellular compartments.

Because both P₄ and PDBu induced redistribution of hOAT4, we then asked whether P₄ inhibited hOAT4 activity through activation of PKC. It was indicated that P₄ may exert its effect through intracellular mechanisms dependent on PKC. However, our results showed that the inhibitory effect of P₄ could not be prevented by pretreating the cells with the PKC inhibitor staurosporine (Fig. 5).

In conclusion, we are first to show that both P₄ and activation of PKC inhibit hOAT4 activity through redistribution of the transporter from cell surface to the intracellular compartments. However, P₄ regulates hOAT4 activity by mechanisms independent of PKC pathway.

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