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

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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 present study, we investigated the effects of 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 Vmax without significant change in Km. 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.

Acute regulation

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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/CaCl₂/MgCl₂ (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 aliquoted for liquid scintillation counting. The uptake count was stan-
dardized 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 V<sub>max</sub> (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 V<sub>max</sub> could be affected by either a reduced number of the transporter at the
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...
Fig. 5. P₄-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 μM, 5 min) followed by incubation with PDBu (10⁻⁶ M, 15 min) in the presence or absence of staurosporine (2 μM). The uptake of [¹H]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 μM, 5 min) followed by incubation with PDBu (10⁻⁶ M, 15 min) in the presence or absence of staurosporine (2 μM). 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. 8 M, 5 min) followed by incubation with PDBu (10⁻⁶ M, 15 min) in the presence or absence of staurosporine (2 μM). The uptake of [¹H]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 ± SE (n = 3). Middle: effect of staurosporine on P₄-induced inhibition of hOAT4 surface expression. BeWo b30-10 Cells stably expressing hOAT4 were pretreated with staurosporine (2 μM, 5 min) followed by incubation with P₄ (10⁻⁶ M, 1 hr) in the presence or absence of staurosporine (2 μM). 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 P₄-treated cells.

It was reported that the plasma P₄ concentration at term is 1.0 × 10⁻⁶ M, and the intracellular P₄ concentrations in placenta were 12 times higher than those in maternal plasma (11). Therefore, 10⁻⁶ M P₄ could be reached in the placenta at term. The plasma E₂ concentrations are around 10⁻⁸ and 10⁻⁷ M during pregnancy (2, 11). However, we did not observe any significant effect of E₂ 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 P₄ (Fig. 2) showed that the reduced transport activity was contributed by a reduced maximum transport velocity (Vₘₐₓ) without affecting the binding affinity (1/Kₘₐₓ) for the substrates. Vₘₐₓ 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 P₄ on hOAT4 expression both at the cell surface and in the total cell lysates. Our results showed that P₄ 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 endocytic 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 endocytic pathway. Whether such a pathway is also involved in progesterone-induced rapid redistribution of hOAT4 needs further investigation.
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 (5). 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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REFERENCES