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Molecular Regulation of Organic Anion Transporting Polypeptide 1A2 (OATP1A2) by Taurocholic Acid in Bewo Cells

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Abstract

To characterize the mechanisms of action of taurocholic acid(TCA) and farnesoid X receptor(FXR) on organic anion transporting polypeptide 1A2(OATP1A2) expression in placental Bewo cell line. Quantitative real-time PCR and Western blots were used to detect OATP1A2 in Bewo cells cultured with TCA and pcDNA3.1(+)-hFXR transfected Bewo cells after incubation with 2mM TCA for 48 hours. TCA(0.02mM) induced the mRNA and protein expression of OATP1A2 by 3 and 1.6 fold ($p<0.05$), respectively, while 0.2 and 2mM TCA induced mRNA and protein expression by 1.5 and 1.3 fold, respectively. The concentration of TCA was negatively correlated with OATP1A2 gene expression ($P<0.05$). In pcDNA3.1(+)-hFXR transfected Bewo cells with 2mM TCA demonstrated a 2-3 fold increase in OATP1A2 over controls ($P<0.05$). TCA is one of the regulation factors for OATP1A2 in the Bewo cell line. A low dose of TCA can induce fetal membrane expression of OATP1A2. This may present a physiological or compensatory mechanism of the placenta, while the high dose of TCA may produce a pathological or pathogenic mechanism. Farnesoid X receptor may act in synergy with TCA to increase the expression of OATP1A2. This may be a treatment strategy for fetal cholestasis.

Key words: Taurocholic acid, Bewo cells, OATP1A2, FXR, bile acids transporter, fetal cholestasis, ICP.

Introduction

Organic anion-transporting polypeptide (OATP) is a membrane transport protein family (1). There are 11 known human OATPs (2). They are expressed in many tissues on basolateral and apical membranes. Anions are transported through mediation of the exchange of extracellular organic anion or bile acid with intracellular HCO^-_3 or glutathione (GSH) (3).

Organic anion transporting polypeptide 1A2 (OATP1A2) is the first anion carrier to be discovered mainly in the liver and localized on the basolateral membrane of the hepatocytes. It is involved in the Na^+-independent bile acid uptake. However, OATP1A2 is believed to behave as a bi-directional transporter (4,5). Ph and GSH could change its expression (4,6).

As well as the liver, OATP1A2 is also found to be one of the bile acid transporters on the basement membrane of human trophoblast(7). Our previous study(8) shows that OATP1A2 mRNA level is up-regulated in placentas of cases with intrahepatic cholestasis of pregnancy (ICP), but the mechanism is unclear. ICP is a common complication during pregnancy, which can be diagnosed by the serum level of total bile acid (TBA). Recently, one prospective randomized controlled test reported that TBA levels of higher than 40μmol/L increases the severity of complications during pregnancy. (9) Because taurocholic acid (TCA) is the most abundant element of TBA in ICP (10,11), we presume that TCA may be an important regulator to OATP1A2 on trophoblasts.

TCA has also been identified as one of the natural ligands for the farnesoid X receptor (FXR, NR1H4), which is also called bile acid receptor because of its sensitivity to changes in bile acid. The EC50 of TCA for activation of FXR is more than 1mM in vitro (12-15). FXR is particularly influential on bile acid metabolism in enterohepatic circulation. In the liver, FXR prevents bile acid accumulation by decreasing both their biosynthesis and uptake, which promotes their excretion in bile. In accordance with this role, the target genes are bile acid receptors. For example, the transcription of bile salt export pump (BSEP), organic solute transporter α/β (Ostα/Ostβ), and OATP8 can be directly activated by FXR binding to an IR-1 element in the promoter area (16,17). Jung et al. reported that OATP8 in hepatocytes was transactivated by FXR when cultured with CDCA (18,19). Miyata and colleagues (2005) (20) found that BSEP mRNA and protein levels increased in the wild-type mice, but decreased in the FXR-null mice, when fed with cholic acid. In addition, higher cholic acid intake increased bile acid output in wild-type mice; meanwhile, higher cholic acid intake decreased bile acid output in FXR-deficient mice. This study demonstrates the FXR-dependent protective mechanism for cholic acid-induced toxicity. Similarly, Liu et al. (2003) (21) found that a ligand for FXR, GW4064, protected the liver from cholestatic injury in the bile duct-ligation and alpha-naphthylisothiocyanate models of cholestasis. FXR is a master regulator of the homeostasis of bile acids and functions as a primary bile acid sensor, which suggests that ligands for FXR could be a new treatment for hepatocholestasis (22,23).

Recent studies indicate that FXR is also expressed in placenta and cytotrophoblasts (24), but whether the function is the same as in the liver is not clear. Since FXR regulates most bile acid transporters with the cooperation of physical ligands such as TCA in the liver

(25,26) and is overexpressed in ICP placentas (27), it may participate as a mediating factor.

The choriocarcinoma Bewo cell line expresses very similar genes as human trophoblast cells. In this study, Bewo cells with or without FXR overexpression were used to study the regulation of OATP1A2 by TCA through FXR in vitro. This might partly explain the etiology and treatment mechanism of fetal cholestasis in ICP.

Materials and methods

Plasmid construction

The expression vector pcDNA3.1(+)-hFXR was constructed by inserting the cDNA fragment encoding the full-length human FXR (NP_005114) into pcDNA3.1(+) at BamHI/XhoI. Primers (forward 5'-GGGGGGATCCGAAAAATTGGGATGG-3'; reverse 5'-CAGCCTCGAGCATCACTGCACGTCC-3') were designed by Primer Premier 5.0 (PREMIER Biosoft International, Canada). The integrity of the sequence for this new construct was confirmed by DNA sequencing. To assess transfection efficiency, pcDNA3.1(+)-RFP was constructed and red fluorescence intensity was observed under a fluorescence microscope. In addition, FXR protein expression was verified by Western blotting.

Cell culture and transfection

BeWo cells (ATCC) were plated in 6-well plates at a density of 1 x 10^4 cells/well and cultured in Kaighn's modification of Ham's F-12 medium (F12k, Gibico) supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C under 5% CO_2. When cells reached 70–80% confluence, taurocholic acid (Fluka, #86339) was added into the medium at concentrations of 0.02, 0.2 and 2mM for 48 hours. To transfect the cells, plasmids were added using lipofectamin 2000(Invitrogen) at a concentration of 25 ng plasmids/well. After 24 hours transfection, cells were incubated with fresh medium with or without 2mM TCA for 48 hours.

Quantitative real-time PCR.

Total RNA was extracted from the cultured cells using TRIzol (Invitrogen). RNA was reversed-transcribed using a Superscript II RNase H-Reverse Transcriptase system (Invitrogen) TaqMan RT-PCRs were performed on the FTC-2000C-II Real-time FQ-PCR system (Funglyn Biotech Co. Ltd, Canada) using primers listed in Table 1. The annealing temperatures of OATP1A2, FXR and β-actin gene were 54℃, 56℃ and 52℃, respectively. The comparative threshold cycle (C_t) method for relative quantification ($2^{-\Delta\Delta C_t}$) was used to quantitate gene expression according to Applied Biosystem recommendations (7900 HT Real-Time fast and SDS enterprise and database user guide). Expression of target genes was normalized to the level of β-actin.

Western Blot

Total cell lysates were prepared by homogenization in RIPA lysis buffer on ice. Insoluble material was removed by centrifugation at 14,000 *g* for 10 min at 4°C. Protein concentration was measured by BCA assay. 50 μg protein was then separated on a 8% sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel and transferred to a nitrocellulose membrane. After blocking with 2% nonfat milk for 3 hours and overnight incubation with primary antibodies (anti-OATP1A2: 1:400, sc-18427, Santa Cruz; anti-β-actin: 1:2000, sc-1616, Santa Cruz), membranes were then washed and incubated with horseradish peroxidase-conjugated rabbit anti-goat antibody and visualized by enhanced chemiluminescence recorded on CP-BU New X-ray film (Agfa, Westerlo-Heultje, Belgium). Band intensity was quantified by densitometry using Quantity One software from Bio-Rad.

Table1. Primers and probe for target genes*

Gene	Sequences
OATP-A (147bp)	F 5′-CCATTGGAACGGGAATAAAC-3′
	R 5′-TGCTCATCGCTGACAAGATT-3′
	P 5′-CAGGTCCTTTGTCGCACAGCC-3′
AE2 (191bp)	F 5′-GAGGCCTTCTTCTCGTTCTG-3′
	R 5′-GTCTCATA GATGAAGATGAGTG-3′
	P 5′-CGGACCAGGAAGCTCCCCTC-3′
MRP2 (174bp)	F 5′-CACCATAAAGGACAACATCCTT -3′
	R 5′-AGGCTGATCCGCTGCTTCTG -3′
	P 5′-CTCTCCTCCCAGACTTGGAAATG -3′
β-actin (114bp)	F 5′-GCCAACACAGTGCTGTCT-3′
	R 5′-AGGAGCAATGATCTTGATCTT-3′
	P 5′-FAM -ATCTCCTTCTGCATCCTGTC- TAMRA-3′

F: forward primer, R: reverse primer, P: TaqMan probe. *designed and synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd, China.

Statistical analysis

All studies were repeated three times and descriptive statistics were performed for each data set. Statistical analysis was performed using Statistical Program for Social Sciences (SPSS) Version 13.0. For expression studies, one-way ANOVA was applied followed by the Student-Newman-Keul's test. Statistical significance was set at $p<0.05$.

Results

Influence of TCA concentration on OATP1A2 gene expression

The effect of TCA concentration on OATP1A2 expression in Bewo cells was assessed through measurements of mRNA and protein expression (Figure 1). At a TCA concentration of 0.02 mM, mRNA expression was increased 2.9 fold and protein expression was increased 1.6 fold ($p< 0.05$). Higher concentrations of TCA had a lower effect on OATP1A2 gene expression; more specifically, TCA concentrations of 0.2 mM and 2 mM only produced a 1.5 fold and 1.3 fold increase in mRNA and protein expression, respectively.

TCA effect on OATP1A2 in hFXR transfected Bewo cells

The effect of TCA on Bewo cells transfected with the reconstructed plasmid pcDNA3.1(+)-hFXR was

also assessed (Figure 2). OATP1A2 mRNA expression increased by 2 fold and protein expression increased by 2.6 fold ($p<0.05$). Treatment of cells transfected by pcDNA3.1(+) with 2mM TCA increased OATP1A2 mRNA expression by 1.3 fold and protein expression by 1.2 fold ($p<0.05$). In contrast, cells transfected with the pcDNA3.1(+)-hFXR without TCA treatment decreased OATP1A2 1.7 fold in mRNA level ($P<0.05$). For this condition, the protein level remained unchanged ($P>0.05$).

Discussion

The purpose of this study was to investigate the relationships between OATP1A2, TCA and FXR, which may create an in vitro model for placental bile acid transportation study and govern the etiology of intrahepatic cholestasis of pregnancy.

Of note, OATP1A2 has been detected in the placenta and fetal liver with a liver-like position and function (24,28). However, the understanding of its effect on bile acid regulation has been unclear. In our study, a low dose (0.02mM) of taurocholic acid (TCA) induces the mRNA expression of OATP1A2 ($P<0.05$). A higher dose (2mM) of TCA, however, only increases OATP1A2 1.3-fold both in mRNA and protein expression, $P<0.05$. Hence, the effect of TCA on OATP1A2 is negatively correlated with concentration ($P<0.05$). This suggests that TCA is an important regulator to OATP1A2 in trophoblasts, which could be a cell model for placental bile acid transportation. Moreover, according to our results, the overexpressed-FXR trophoblast cells express

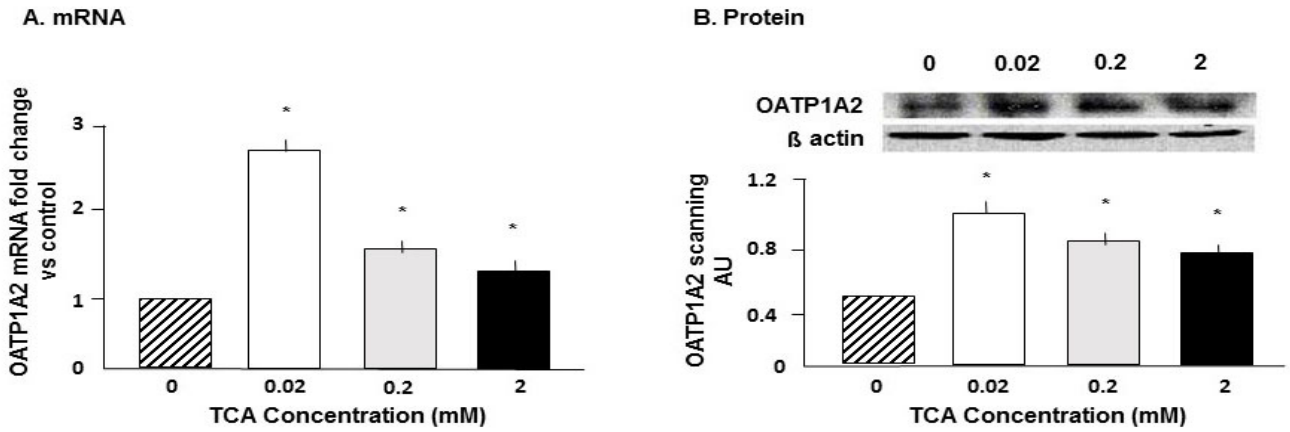

Figure1. Dosage dependent regulation of TCA on OATP1A2 expression in Bewo cells**. A**: RT-PCR was used to detect OATP1A2 mRNA expression in Bewo cells treated with 0, 0.02, 0.2, 2mM TCA for 48 hours. Results calculated as fold changes vs. control were normalized by actin mRNA levels. Data are shown as mean±SE. One-way ANOVA was applied followed by the Student-Newman-Keul's test. *$p<0.05$ $n=3$ in each group. **B**: Western blot was used to compare OATP1A2 protein levels in Bewo cells treated with 0, 0.02, 0.2, 2mM TCA for 48 hours and quantified AU by Quantity One software from Bio-Rad. Data were normalized by βactin protein levels and shown as mean±SE. One-way ANOVA was applied followed by the Student-Newman-Keul's test. *$p<0.05$, $n=3$ in each group.

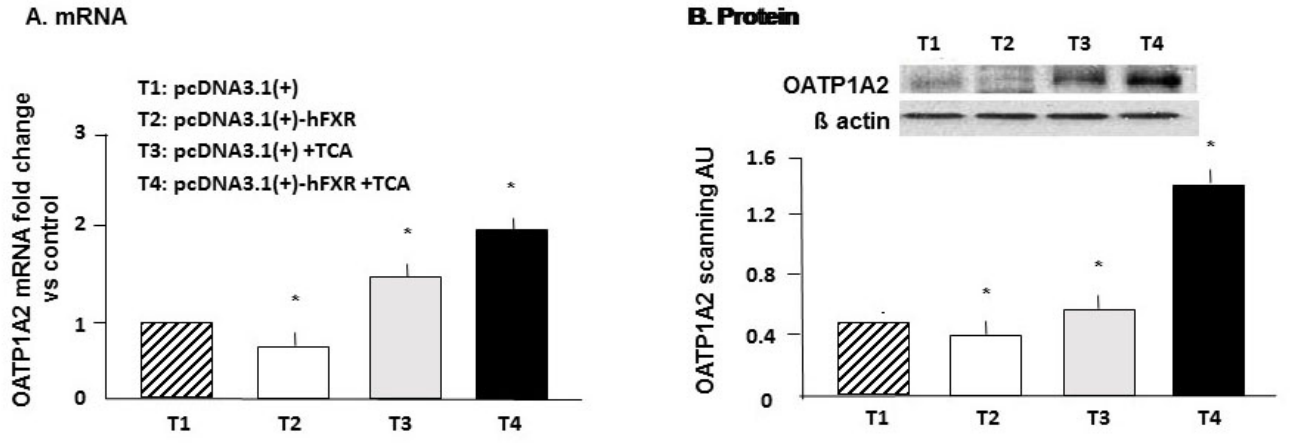

Figure2. OATP1A2 expression in FXR-overexpressed Bewo cells treated by TCA**. A**: RT-PCR was used to detect OATP1A2 mRNA expression in pcDNA3.1(+) or pcDNA3.1(+)-hFXR transfected Bewo cells treated with or without 2mM TCA for 48 hours. Results calculated as fold changes vs. control were normalized by actin mRNA levels. Data are shown as mean±SE. One-way ANOVA was applied followed by the Student-Newman-Keul's test. *$p<0.05$, $n=3$ in each group. **B**: Western blot was used to compare OATP1A2 protein levels in pcDNA3.1(+) or pcDNA3.1(+)-hFXR transfected Bewo cells treated with or without 2mM TCA for 48 hours. Results were quantified with AU by Quantity One software from Bio-Rad. Data were normalized by βactin protein levels and shown as mean±SE. One-way ANOVA was applied followed by the Student-Newman-Keul's test. *$p<0.05$, $n=3$ in each group.

T1: Bewo cells trasfected by pcDNA3.1(+) and cultured without TCA;

T2: Bewo cells transfected by pcDNA3.1(+)-hFXR and cultured without TCA;

T3: Bewo cells transfected by pcDNA3.1(+) and cultured with 2mM TCA;

T4: Bewo cells transfected by pcDNA3.1(+)-hFXR and cultrured with 2mM TCA.

more OATP1A2 than the non-transfected ones when treated by high concentration TCA. This suggests that FXR participates as a mediating factor in TCA induced OATP1A2 up-regulation in placental bile acid transportation which is similar as in liver.

These regulations between OATP1A2, TCA and FXR may underpin the etiology of intrahepatic cholestasis of pregnancy (ICP). Our previous studies (8,27) show that OATP1A2 and FXR levels are up-regulated in placentas of ICP cases when compared to placentas from normal pregnancies. An increase in TCA is also representative of an increase in TBA (10,11,), which is the gold standard for ICP diagnosis and closely negatively correlated with fetal outcomes. As described in Figure 3, our study shows that a lower concentration of TCA (0.2 mM) elevates the expression of OATP1A2 more than a higher concentration of TCA (2 mM). This may indicate that the mild increase of TCA induces the expression of OATP1A2 on the fetal membrane, which could up-regulate the bile acid flow from fetus to mother. This could be a physiological or compensatory mechanism of placenta in normal pregnancy. The compensatory function, however, will be weakened with the increasing concentration of TCA. As a result, the bile acid flow from the fetus to the mother may be down-regulated, which may lead to the development of fetal cholestasis. Therefore, OATP1A2 and TCA may play important roles in pathological or pathogenic mechanisms in ICP. FXR overexpression can increase bile acid transport through an increase of OATP1A2 expression, which may be an approach to treat fetal cholestasis. Validation of such an approach may further motivate the use of FXR ligands to treat both maternal and fetal cholestasis. Since OATP1A2 is a bi-directional transporter, exploring the direction of the TCA transport will further support our findings.

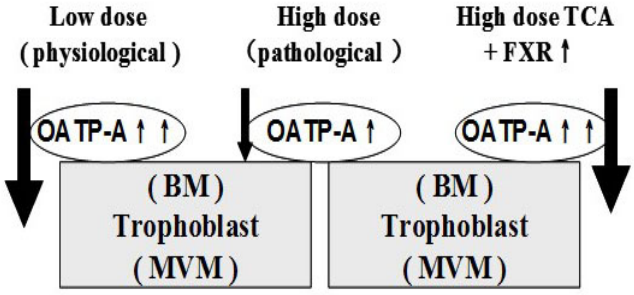

Figure3. Mechanism hypothesis of fetal bile acid flow regulation on maternal-fetal interface by TCA-FXR-OATP1A2 model. OATP-A: organic anion transporting polypeptide 1A2(OATP1A2); BM: basement membrane; MVM: microvillous membrane

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