Regulation of metabolizing enzymes and transporters for drugs and bile salts in human and rat intestine and liver

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Chapter 6

Expression and Regulation of The Bile acid Transporter OSTα/OSTβ, in Rat and Human intestine and Liver

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Abstract

The regulation of the OSTα and OSTβ expression was studied in the rat jejunum, ileum, colon and liver and in human ileum and liver by ligands for the farnesoid X receptor (FXR), pregnane X receptor (PXR), vitamin D receptor (VDR) and glucocorticoid receptor (GR) using precision-cut tissue slices. The gradient of protein and mRNA expression for rOSTα and rOSTβ in segments of the rat intestine paralleled that of rASBT. OSTα and OSTβ mRNA expression, quantified by qRT-PCR in rat jejunum, ileum, colon and liver, and in human ileum and liver was positively regulated by FXR and GR ligands. In contrast, the VDR ligand, $1,25(OH)_2D_3$ decreased the expression of rOSTα-rOSTβ in rat intestine, but had no effect on human ileum, and rat and human liver slices. Lithocholic acid (LCA) decreased the expression of rOSTα and rOSTβ in rat ileum but induced OSTα-OSTβ expression in rat liver slices, and human ileum and liver slices. The PXR ligand, pregnenolone-16α carbonitrile (PCN) had no effect. This study suggests that, apart from FXR ligands, the OSTα and OSTβ genes are also regulated by VDR and GR ligands and not by PXR ligand. This study further shows that VDR ligands exerted different effects on OSTα-OSTβ in the rat and human intestine and liver compared with other nuclear receptors, FXR, PXR, and GR, pointing to species- and organ-specific differences in the regulation of OSTα-OSTβ genes.

Keywords: OSTα-OSTβ, regulation, nuclear receptors, intestinal slices, liver slices

Introduction

Bile acids (BA) undergo extensive enterohepatic cycling and are actively reabsorbed in the terminal part of the ileum, the bile duct epithelial cells (BEC) (14) and the renal proximal tubular cells (6, 42). They play an important role in the regulation of bile acid synthesis and cholesterol homeostasis. The primary transporter involved in the absorption of bile acids is the sodium dependent bile acid transporter, ASBT (SLC10A2) (33), that is expressed along the apical surface of ileocytes, BEC and renal proximal tubular cells. In enterocytes, bile acids are effluxed out of the cells into the portal circulation, and may be transported back to the intestinal lumen. Several basolateral bile acid transporters such as truncated ASBT (tASBT), MRP3 and MRP4, showing affinity towards bile acid transport have been proposed (11, 16, 29, 35). Although MRP3 was shown to transport bile salts and regulated by chenodeoxycholic acid (CDCA) in human ileum (11), its role in ileal bile salt absorption may not be significant, since $mrp3^{-/-}$ mice failed to show any apparent defect in bile acid absorption (43).

Recently, Wang et al. (41) identified an organic solute transporter (OST) consisting of two half transporters, α and β (OSTα and OSTβ) in the skate, Raja Ernacea. Subsequently, rodent and human OSTα-OSTβ orthologues that are able to mediate sodium independent transport of organic anions, bile acids and sterols in transfected Xenopus Oocytes were identified (1). The expression of OSTα and OSTβ are shown to parallel that of ASBT expression in enterocytes along the length of the intestine and were co-incident with ASBT in BECs and renal proximal tubular cells of rat, mouse and human (1, 7). The OSTα and OSTβ proteins are found to be localized at the basolateral membrane and catalogued as the ileal bile acid basolateral transporter in the mouse (7), since bile acid homeostasis was perturbed in the Ostα knockout mouse (2, 28). The mouse and human OSTα-OSTβ genes are regulated by the farnesoid X receptor (FXR) and the liver X receptor α (LXRα) (9, 15, 24). Both FXR and LXRα heterodimerize with the retinoic acid X receptor α (RXRα), and, upon ligand binding, the resulting complex binds to the inverted repeat-1 (IR1) in the promoters of OSTα and OSTβ, thereby increasing their expression. Furthermore, human and mouse OSTα and OSTβ promoters are endowed with binding sites for the transcription factors, hepatocyte nuclear factor 4α (HNF4α) (24) and liver receptor homolog protein-1 (LRH-1) (9, 17).

Studies on rodent and human OSTα-OSTβ genes in the intestine and liver usually entail use of FXR and LXRα ligands on immortalized cell lines such as CT26, Caco-2, Huh-7 and HepG2 cells (3, 15, 24). However, these cell lines lack the normal expression of various nuclear receptors, transporters and coactivators, and are unable to reflect the regulation in distinct segmental regions of OSTα and OSTβ genes in intestine. In the rat, the regulation of rOSTα-rOSTβ genes has not been studied in great detail. Landrier et al., (15) reported on the induction of hOSTα and hOSTβ genes by CDCA, the FXR ligand, in human ileum biopsies after 4 hours in culture. However, evidence for the regulation of

hOSTα-hOSTβ in human livers was predominantly obtained indirectly from analysis of the livers of patients with cholestatic disease (3, 45). In the mouse *in vivo*, the regulation of Ostα-Ostβ by FXR in the intestine was shown (9). In this study, we investigated whether OSTα and OSTβ genes were regulated by ligands for the vitamin D receptor (VDR) and glucocorticoid receptor (GR) in the rat and human liver and intestine, since these nuclear receptors were reported to regulate ASBT (5, 12, 23), the bile acid transporter that was under negative regulation by FXR in mouse, rabbit and human but not in rat intestine (4, 18, 22). Precision-cut tissue slices were used from the rat intestine (jejunum, ileum and colon) and liver and human ileum and liver, and the effects of VDR and GR ligands are compared with those of FXR on the regulation of the mRNA expression of the OSTα and OSTβ genes. In addition, the involvement of PXR in the regulation of OSTα and OSTβ genes was also investigated. This *ex-vivo* model enables to study the regulation of genes of interest under controlled and nearly physiological conditions directly, and allows the comparison of direct effects of ligands in different organs under identical conditions (30, 38).

Materials and methods

Male Wistar (HsdCpb:WU) rats weighing about 230 - 250 g were purchased from Harlan (Horst, The Netherlands). Pieces of human liver and ileum tissue were obtained as surgical waste from the University Medical Center, Groningen (UMCG) with the informed consent of the patients/donors. $1,25(OH)_2D_3$ in ethanol was purchased from BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA. Chenodeoxycholic acid (CDCA) and lithocholic acid (LCA) were purchased from Calbiochem, San Diego, California, Dexamethasone was obtained from Genfarma bv, Maarssen. Ethanol, methanol and DMSO were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO); gentamicin sulfate and Williams medium E with glutamax-I and amphotericin B (Fungizone)-solution were obtained from Gibco (Paisley, UK). D-Glucose and HEPES were from ICN Biomedicals, Inc. (Eschwege, Germany). University of Wisconsin organ preservation solution (UW) was obtained from Du Pont Critical Care, Waukegab, Illinois, U.S.A. Low gelling temperature agarose, budisonide (BUD) and pregnenolone-16α carbonitrile (PCN) were purchased from Sigma – Aldrich (St. Louis, MO). RNAeasy mini columns were obtained from Qiagen, Hilden, Germany. Random primers (500 μg / ml), $MgCl_2$ (25 mM), RT buffer (10X), the PCR nucleotide mix (10 mM), AMVRT (22 U/μl) and RNasin (40 U/μl) were purchased from Promega Corporation, Madison WI, U.S.A. Assay-on-Demand™ human GAPDH primers and probe for the Taqman analysis were purchased from Applied Biosystems, Warrington, UK. All SYBR Green primers were purchased from Sigma Genosys. The Taq Master Mixes was procured from Eurogentech. The rabbit anti-rat Ostα and Ostβ antibodies are generous gifts from Dr. Ned Ballatori (Rochester, New York, U.S.A). The secondary antibody, Alexa Fluor-488 anti-rabbit immunoglobulin (IgG) was purchased from Invitrogen, Moleuclar Probes, Eugene, OR, U.S.A. All reagents and materials used were of the highest purity that is commercially available.

Experimental protocols. All experimental protocols involving animals were approved by the Animal Ethical Committee of the University of Groningen. Experimental protocols involving human tissue (liver and ileum) were approved by the Medical Ethical Committee of the UMCG.

Preparation of rat and human intestinal and liver slices. The small intestine, colon and liver were excised from the rat under isoflurane/O_2 anaesthesia. The small intestine and colon were immediately placed into ice-cold Krebs-Henseleit buffer supplemented with 10 mM HEPES, 25 mM sodium bicarbonate and 25 mM D-glucose, pH 7.4 (KHB), saturated with carbogen (95%O_2/5%CO_2) and stored on ice until preparation of the slices. The rat liver was stored in ice-cold UW until slicing. Pieces of human liver tissue were obtained from patients undergoing partial hepactectomy for the removal of carcinoma (PH livers) or from redundant parts of donor livers remaining after split-liver transplantation (Tx livers) as described previously by Olinga et al. (26). Human ileum tissue was obtained as a part of the surgical waste after resection of the ileo-colonic part of the intestine in colon carcinoma patients. After surgical resection, the ileum tissue was immediately placed in ice-cold carbogenated KHB. Human liver and ileum donor characteristics are as reported earlier (13). Human liver and ileum slices were prepared within 30 to 60 min after resection. Rat and human intestinal and liver slices were prepared according to the published methods (8, 26, 40).

Induction studies. Precision-cut slices, prepared from rat intestine (jejunum, ileum and colon) and human ileum were incubated individually in 12-well sterile tissue culture plates (Grenier bio-one GmbH, Frickenhausen, Austria) containing 1.3 ml Williams medium E supplemented with D-glucose (final concentration of 25 mM), gentamicin sulfate (50 µg/ml) amphotericin / fungizone, (250 µg/ml) and saturated with carbogen. The plates were placed in humidified plastic container kept at 37° C and continuously gassed with carbogen and shaken at 80 rpm. Intestinal slices were incubated with 1,25$(OH)_2D_3$ (final concentrations of 5 nM, 10 nM and 100 nM), CDCA (final concentration of 50 µM), LCA (final concentrations of 5 µM and 10 µM), DEX (final concentrations of 1 µM and 50 µM), BUD (final concentration of 10 nM) and PCN (final concentration of 10 µM) added as a 100-times concentrated stock solution in ethanol (1,25$(OH)_2D_3$), methanol (CDCA and LCA) or DMSO (DEX, BUD and PCN). Higher concentrations of CDCA (100 µM) and LCA (50 µM) were toxic to the intestinal slices. Rat intestinal slices were incubated for 12 h, since at 24 h, the expression of villin was found to be decreased. Human ileum slices were incubated for 8 h and 24 h; villin expression was stable up to 24 h. Data are presented for 24 h only, since the results obtained at 24 h were not different from those obtained at 8 h. Further, rat ileum slices were incubated in presence of both 1,25$(OH)_2D_3$ (final concentration of 100 nM) and CDCA (final concentration of 50 µM). Control slices were incubated in Williams medium E (supplemented with D-glucose and gentamicin sulfate) with 1% ethanol, methanol, DMSO and ethanol + DMSO without ligands. From a single rat or human tissue sample, six (rat intestine) or three (human ileum) replicate slices were

subjected to each experimental condition. After the incubation, these replicate slices were harvested, pooled and snap-frozen in liquid nitrogen to obtain sufficient total RNA for quantitative real time PCR (qRT-PCR) analysis. Samples were stored at – 80° C until RNA isolation. These experiments were replicated in 3-5 rats and 3-5 human ileum donors.

Liver slices (8 mm diameter and 250 µm thick) were incubated individually in sterile six-well tissue culture plates (Grenier bio-one GmbH, Frickenhausen, Austria) containing 3.2 ml Williams medium E supplemented with D-glucose to a final concentration of 25 mM, gentamicin sulfate (50 µg/ml) and saturated with carbogen. The plates were placed in humidified plastic container kept at 37° C and continuously gassed with carbogen and shaken at 80 rpm. Liver slices were induced with $1,25(OH)_2D_3$ (final concentration, 100 nM), CDCA (final concentration, 100 µM), LCA (final concentration, 50 µM), DEX (final concentration, 50 µM), BUD (final concentrations 10 nM and 100 nM) and PCN (final concentration, 10 µM) added as a 100-fold concentrated stock solution in ethanol (for $1,25(OH)_2D_3$), methanol (for CDCA and LCA) or DMSO (DEX, BUD and PCN). Rat and human liver slices were incubated for 8 h and 24 h. Data are presented for the 24 h time point, since the results were similar to those obtained at 8 h. Control liver slices were incubated in supplemented Williams medium E with 1% ethanol, methanol and DMSO without inducers. From a single rat / single human liver donor, three replicate slices were subjected to identical incubation conditions. At the end of the incubation these replicate slices were harvested, pooled and snap-frozen in liquid nitrogen to obtain sufficient total RNA for qRT-PCR analysis. Samples were stored at – 80° C until RNA isolation. These experiments were replicated in 3-5 rats and 4-5 human liver donors.

RNA isolation and qRT-PCR. Total RNA from rat and human intestine and liver samples was isolated using RNAeasy mini columns from Qiagen according to the manufacturer's instruction. The RNA concentration and quality were determined by measuring the absorbance at 260 nm, 230 nm and 280 nm using a Nanodrop ND100 spectrophotometer (Wilmington, DE USA). The ratios of absorbance measured at 260 over 280 and 230 over 260 were found to be above 1.8. About 2 µg of total RNA in 50 µl was reverse-transcribed into template cDNA as reported earlier by van de Kerkhof et al. (39).

qRT-PCR for the rat and human genes of interest was performed using primer sequences listed in Table 1 by two detection systems, SYBR Green or Taqman® analysis according to the availability of primer sets. All primer sets were analyzed using BLASTn to ensure primer specificity for the gene of interest **(http://www.ncbi.nlm.nih.gov/BLAST/)**. For the SYBR Green, ~ 50 ng of cDNA was used in a total reaction mixture of 20 µl. For the Taqman® analysis ~ 250 ng of cDNA was used in a total reaction mixture of 10 µl. The PCR conditions were similar to those described in an earlier report (13). All samples were analyzed in duplicates in 384 well plates using ABI7900HT from Applied Biosystems. Appropriate controls, consisting of water (with water instead of total mRNA, which has been subjected to the reverse

transcription protocol) and the mRNA control (isolated mRNA which has not been subjected to reverse transcription protocol) were subjected to qRT-PCR to determine potential primer dimer formation and contamination of DNA in the isolated samples, respectively. None of the primers showed dimer formation. In addition total RNA from the samples for the preparation of cDNA appeared to be free of DNA contamination. Dissociation curves showed a single homogenous product. The comparative threshold cycle (C_T) method (31) was used for relative quantification, where C_T is inversely related to the abundance of mRNA transcripts in the initial sample. The mean C_T of the duplicate measurements was used to calculate the difference between the C_T for the gene of interest and that of the reference gene (villin for intestine and GAPDH for liver) (ΔC_T), which was compared to the corresponding ΔC_T of the solvent control ($\Delta\Delta C_T$). Data are expressed as fold induction or repression of the gene of interest according to the formula $2^{-(\Delta\Delta CT)}$.

Immunolocalization of OSTα and OSTβ in rat intestine and liver. The rat intestine was washed with 0.9% saline and cut into small pieces. The intestinal tissue was filled with Tissue Tek (Sakura Finetek Europe, The Netherlands), then quickly frozen in cold isopentane (kept at – 80° C) and stored at – 80° C. Sections of 5 µm were cut in a cryostat (Lieca CM 3050) at – 20° C and placed on superfrost plus slides (Menzel, Braunchweig, Germany). Indirect immunofluorescence detection was performed using Ostα and Ostβ antibodies according to the protocol described previously (1). In brief, tissue sections were fixed with acetone cooled to – 20° C for 10 min. Nonspecific binding sites were blocked with 1% bovine serum albumin (BSA) in phosphate buffered saline containing 0.05 % Triton X 100. Primary antibodies were diluted in the blocking buffer, Ostα (m315) (1:200) and Ostβ (mB90) (1:150), and incubated with the sections for 2 h at room temperature. Subsequently, the sections were incubated with the secondary antibody (Alexa Flour-488) at a dilution of 1:50 in blocking buffer for 1 h at room temperature.

Data analysis. All values were expressed as the mean ± S.D. All data (fold-induction and $\Delta\Delta C_T$) were analyzed by paired student's *t*-test using SPSS Version 16 for significant differences between the means. The *P* value < 0.05 was considered as significant.

Table 1 Sequence of oligonucleotides for quantitative Real-Time PCR, rat and human genes (SYBR and Taqman® analysis).

Gene	Forward primer (5' – 3')	Reverse primer (5' – 3')	Gene bank number
r Villin	GCTCTTTGAGTGCTCCAACC	GGGGTGGGTCTTGAGGTATT	XM_001057825
r GAPDH	CTGTGGTCATGAGCCCCTCC	CGCTGGTGCTGAGTATGTCG	XR_008524
r Ostα	CCCTCATACTTACCAGGAAGAAGCTAC	CCATCAGGAATGAGAAACAGGC	XM_221376
r Ostβ	TATTCCATCCTGGTTCTGGCAGT	CGTTGTCTTGTGGCTGCTTCTT	XM_238546
r SHP	CTATTCTGTATGCACTTCTGAGCCC	GGCAGTGGCTGTGAGATGC	NM_057133
h Villin	CAGCTAGTGAACAAGCCTGTAGAGGAGC	CCACAGAAGTTTGTGCTCATAGGC	NM_007127
*h GAPDH	Assay-by-Design™ ID - Hs99999905_m1 (Applied Biosystems)		NM_002046
	6FAM – GCGCCTGGTCACCAGGGCTGCTTTT - NFQ		
*r ASBT	ACCACTTGCTCCACACTGCTT	CGTTCCTGAGTCAACCCACAT	U07183
	Probe - 6FAM - CTTGGAATGCCCCTTTGCCTCT-TAMRA		
*h OSTα	AGATTGCTTGTTCGCCTCC	TCACCACTTGGGGATCATTT	NM_152672
	Probe - 6FAM - CTCAAGTGATGAATTGCCACCTCCTCATACTGG-TAMRA		
*h OSTβ	CAGGAGCTGCTGGAAGAGAT	GACCATGCTTATAATGACCACCA	NM_178859
	Probe - 6FAM - CGTGTGGAAGATGCATCTCCCTGGAATCATTC-TAMRA		

r ,rat genes; h, human genes; primer sets for rat Taqman® Gene analysis* primer sets for human Taqman® Gene analysis.*

Results

Expression of rASBT, rOSTα and rOSTβ in rat intestine and liver. The mRNA expression of rASBT, rOSTα and rOSTβ genes was clearly detectable, not only in rat ileum but also in the jejunum and colon by qRT-PCR (Fig. 1). Expressions of rASBT, rOSTα and rOSTβ mRNA were significantly higher in rat ileum (average threshold cycles (C_T) 23 for rASBT, 17 for rOSTα and 16.5 for rOSTβ) compared to those for the jejunum (29 for rASBT, 20 for rOSTα and rOSTβ) and colon (30 for rASBT, 24 for rOSTα and 22 for rOSTβ). There was no difference between the C_T values in the tissue and those in the slices at the start of the incubation. The gradient in expression of rOSTα, based on the ΔC_T values relative to villin (jejunum:ileum:colon = 1:3.8:0.2), was different from that of rOSTβ (jejunum:ileum:colon = 1:8:0.9) and rASBT (jejunum: ileum:colon = 1:130:4) (Fig. 1). In rat liver, the average threshold cycles (C_T) for rOSTα (30) and rOSTβ (33) were much higher than in intestine. These distributions were further confirmed at the protein level by immunohistochemistry (Fig. 2). In the rat intestine, both rOSTα and rOSTβ were detected at the basolateral membrane of the epithelial cells in all regions of the intestine (Fig. 2). As expected, the highest expression was detected in ileum and a low but clearly detectable expression was observed in colon and to a lesser extent in jejunum. In addition, a decreasing expression from the tip of villus to the crypts was found. In the rat liver, rOSTα - rOSTβ was visibly detectable at the basolateral membrane of the BEC of the larger bile ducts and only a low expression was observed at the basolateral membrane of the hepatocyte.

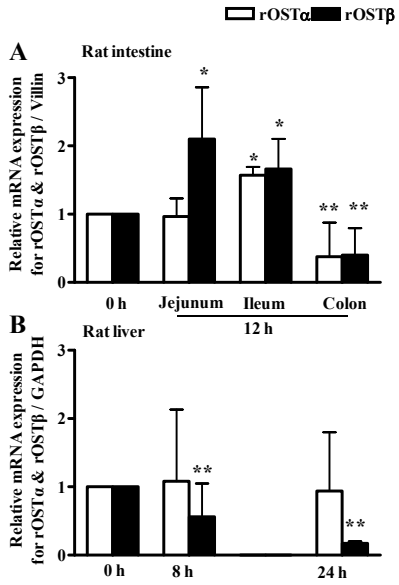

Figure 1. mRNA expression of rASBT, rOSTα and rOSTβ transporters relative to villin expression in intestinal tissue (jejunum, ileum and colon) of the Wistar rat. The average threshold cycles (C_T) for rASBT in jejunum was 29, in ileum 23 and in colon 30.The average C_T for rOSTα and rOSTβ in jejunum was 20, in ileum 17 and 16.5 respectively, and in colon 24 and 22 respectively. The mRNA expression of rASBT, rOSTα and rOSTβ transporters relative to villin in ileum and colon was expressed relative to that in the jejunum, which was set to unity. Each bar represents the results of three animals (n = 3) ± S.D. "" indicates P < 0.05; and "**" indicates P < 0.001.*

Figure 2

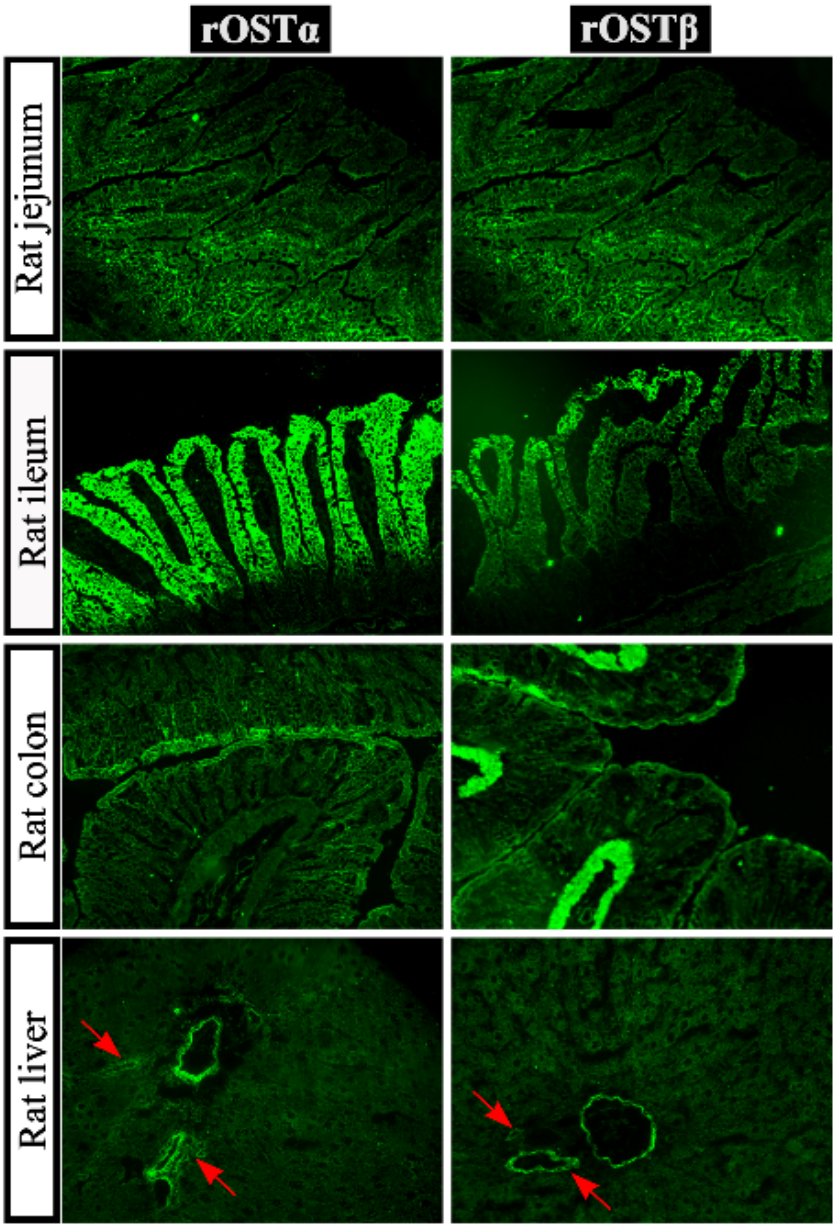

Figure 2. Indirect immunofluorescence showed that rOSTα and rOSTβ were detected at the basolateral surface of the enterocytes in the jejunum, ileum and colon of the Wistar rat. In the liver, rOSTα and rOSTβ proteins are predominantly localized in the bile duct epithelial cells and a low expression was observed in the hepatocytes. Control rat intestinal and liver sections incubated without primary antibodies for rOSTα and rOSTβ did not show any fluorescence (results not shown).

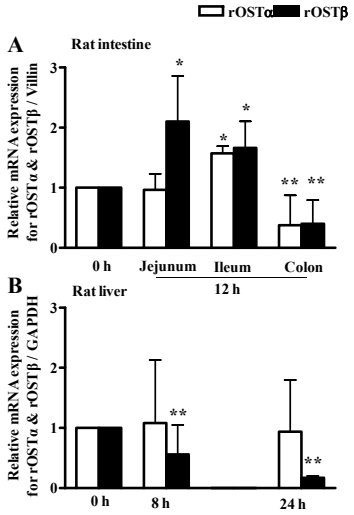

Figure 3. The effect of incubation at 37ºC on rat jejunum, ileum, colon (12 h) and liver slices (8 h and 24 h) on the expression of rOSTα and rOSTβ genes. The mRNA expression of rOSTα and rOSTβ genes relative to villin (intestine) and GAPDH (liver) was quantified by real-time PCR and expressed with respect to the control slices without incubation (0 h) for each of the intestinal segment, which were set to unity. Results are expressed as mean ± S.D. of 4-5 rats. "" indicates P < 0.05; and "**" indicates P < 0.001.*

Regulation of rOSTα and rOSTβ in rat intestine and liver by bile acids. The C_T values of rOSTα and rOSTβ genes were not affected during the preparation of slices and similar C_T values were found in the slices at the start of the incubation and in freshly isolated tissue (data not shown). However, the expression of rOSTα and rOSTβ genes was significantly altered during incubation of slices at 37°C, but the effects differed in different segments of the rat intestine and liver slices (Figs. 3A and B). Incubation of rat jejunum slices for 12 h, either in the absence or presence of various solvents, did not alter the expression of rOSTα but rOSTβ expression was significantly induced (Fig. 3A). In rat ileal slices, the expression of both rOSTα and rOSTβ were significantly elevated (1.5- to 2-fold) (Fig. 3A). In rat colon slices, the expression of both rOSTα and rOSTβ was significantly decreased (3-fold) during incubation (Fig. 3A). In rat liver slices, the mRNA expression of the rOSTα was not altered, whereas rOSTβ was significantly down-regulated during 24 h of incubation (4-fold) regardless of the solvent used (Fig. 3B).

The FXR ligand, CDCA, moderately induced (1.5- to 2-fold) the expression of rOSTα and rOSTβ in the jejunum, ileum and liver, compared with solvent treated control slices (Figs. 4A, B and D). The induction of rOSTα and rOSTβ was dramatically higher in the colon, amounting to 25-fold for rOSTα and 45-fold for rOSTβ (Fig. 4C). In contrast, incubation of rat intestine (jejunum, ileum and colon) and liver slices with LCA, an FXR ligand, with affinity towards VDR (20), exhibited VDR dependent regulation of CYP3A isozymes (21), displayed different effects on rOSTα and rOSTβ genes. LCA significantly decreased the expression of rOSTα and rOSTβ in the ileum (Fig. 4B), and rOSTα expression in the rat jejunum without affecting rOSTβ expression (Fig. 4A). In rat colon, LCA showed a strong, significant up-regulation (up to 10-fold) of the rOSTβ gene without significantly affecting the rOSTα gene (Fig. 4C). In liver slices, a small but significant

(1.5-fold) up-regulation of the rOSTα expression was found in the presence of LCA, whereas rOSTβ expression was decreased (Fig. 4D). Furthermore, both CDCA and LCA significantly induced the SHP expression (~ 2-fold induction) in slices of all regions of the rat intestine and liver (for the effect of CDCA on ileum, see Fig. 6B; data not shown for other tissues). This observation on the up-regulation of SHP was expected for FXR ligands upon incubation with bile salts, and confirms that the FXR pathway was intact in the slices, since these were able to respond to bile salts as signalling agents of FXR.

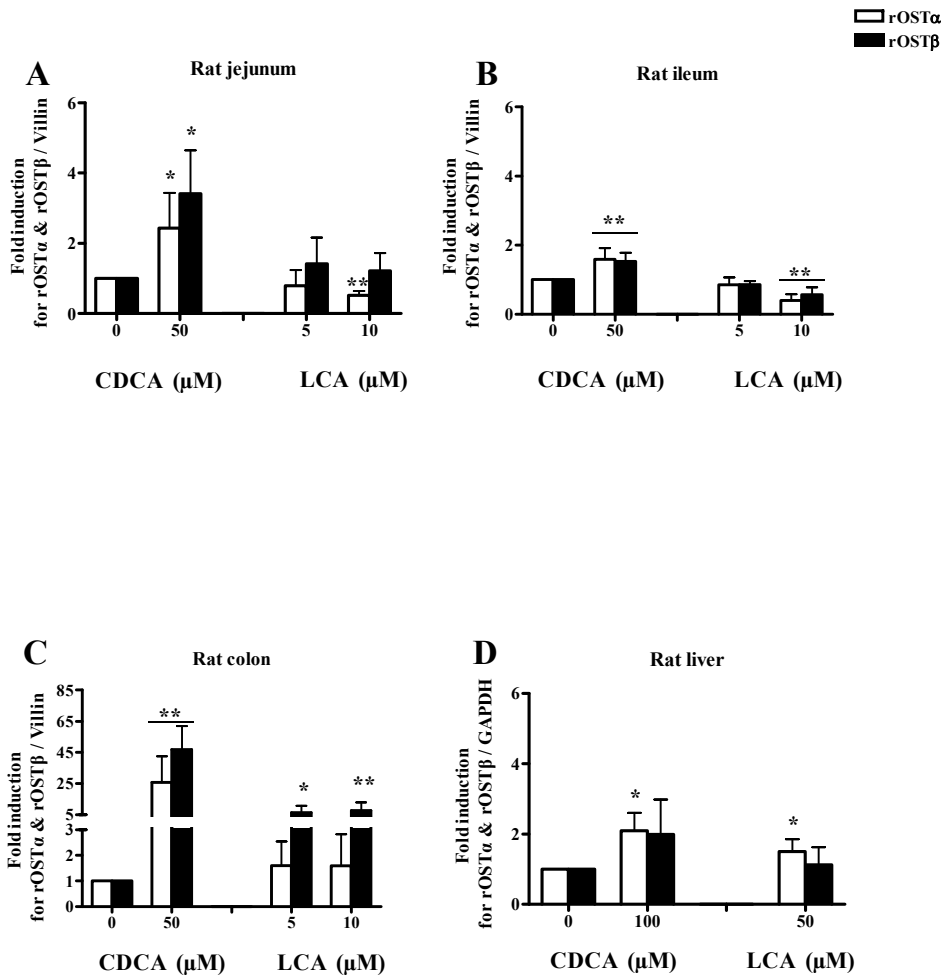

Figure 4. rOSTα and rOSTβ genes are induced by FXR ligands. Rat intestine slices (jejunum (A), ileum (B) and colon (C)) were treated with CDCA (50 μM) and LCA (5 μM and 10 μM) for 12 h, and liver slices (D) were treated with 100 μM of CDCA and 50 μM of LCA for 24h. The mRNA expression of rOSTα and rOSTβ genes relative to villin (intestine) and GAPDH (liver) was quantified by real-time PCR and expressed with respect to the solvent treated controls, which were set to unity. Results are expressed as mean ± S.D. of 4-5 rats. "" indicates P < 0.05; and . "*" indicates P < 0.05; and "**" indicates P < 0.001.*

Regulation of rOSTα and rOSTβ in rat intestine and liver by the VDR ligand. Incubation of rat intestinal slices in presence of the VDR ligand, $1,25(OH)_2D_3$, resulted in a parallel decrease in expression of rOSTα and rOSTβ in jejunum, ileum and colon in a dose-dependent manner (Figs. 5A, B and C). Furthermore, $1,25(OH)_2D_3$ significantly induced the expression of CYP3A1 (> 1000-fold induction), a VDR responsive gene in slices of all regions of the rat intestine (13). The up-regulation of CYP3A1 by $1,25(OH)_2D_3$ confirmed that the slices were able to respond to the VDR ligand. In contrast, incubation of liver slices in the presence of 100 nM $1,25(OH)_2D_3$ did not affect rOSTα and rOSTβ expression (Fig. 5D) but at the same time induced VDR mRNA expression, as expected (unpublished observation). Rat ileal slices, when co-incubated with $1,25(OH)_2D_3$ (100 nM) and CDCA (50 μM), showed significant down-regulation of rOSTα and rOSTβ (fold decrease - rOSTα 0.5 and of rOSTβ 0.7; $P < 0.05$); the observations were identical to those for $1,25(OH)_2D_3$ incubation alone (0.5-fold decrease of rOSTα and 0.7-fold of rOSTβ; $P < 0.05$) and contrasted those for CDCA, which induced rOSTα and rOSTβ (1.5-fold induction of rOSTα and 1.55-fold of rOSTβ; $P < 0.05$) (Fig. 6A). SHP expression was induced by CDCA (fold induction, 2.2; $P < 0.001$) but not by $1,25(OH)_2D_3$ (Fig. 6B).

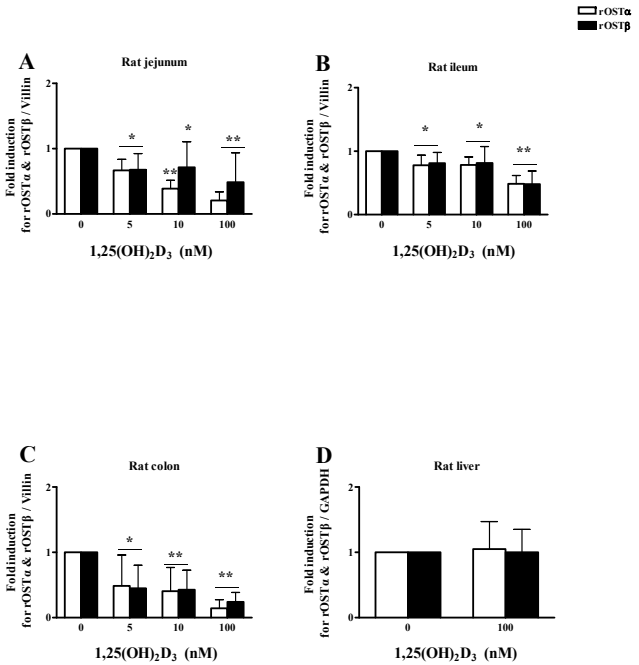

Figure 5. The VDR ligand, $1,25(OH)_2D_3$ decreases the expression of rat rOSTα and rOSTβ genes in jejunum (A), ileum (B), colon (C) and liver (D) slices. Rat jejunum, ileum and colon slices were treated with 5 nM, 10 nM and 100 nM of $1,25(OH)_2D_3$ for 12 h. Rat liver slices were treated with 100 nM of $1,25(OH)_2D_3$ for 24 h. The mRNA expression of rOSTα and rOSTβ genes relative to villin (intestine) and GAPDH (liver) were quantified by real-time PCR and expressed with respect to the solvent treated controls, which were set to unity. Results are expressed as mean ± S.D. of 4-5 rats. "" indicates $P < 0.05$; and "**" indicates $P < 0.001$*

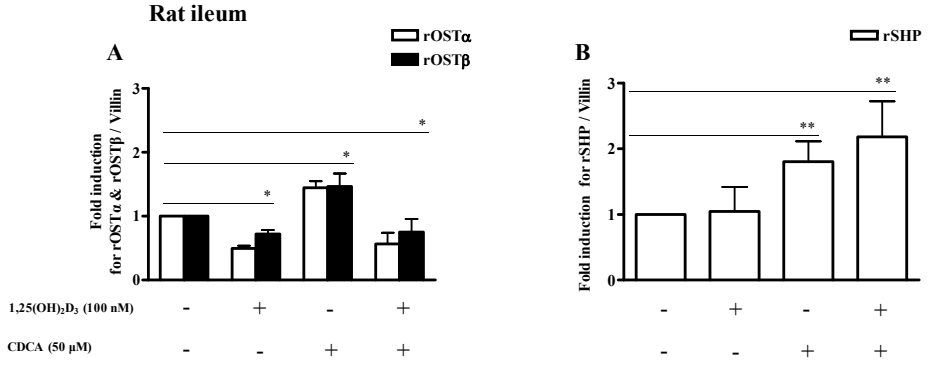

Figure 6. The VDR ligand, 1,25(OH)$_2$D$_3$, decreases the expression of rat rOSTα and rOSTβ genes in ileum, also in the presence of FXR ligand, CDCA (A). 1,25(OH)$_2$D$_3$ did not affect the induction of SHP by CDCA in ileum slices (B). Rat ileum slices were treated with 100 nM of 1,25(OH)$_2$D$_3$, 50 μM of CDCA and 100 nM of 1,25(OH)$_2$D$_3$ + 50 μM of CDCA for 12 h. The mRNA expression of rOSTα and rOSTβ and short heterodimer protein (SHP) genes relative to villin were quantified by real-time PCR and expressed with respect to solvent treated controls, which were set to unity. Results are expressed as mean ± S.D. of 4-5 rats. "" indicates P < 0.05; and "**" indicates P < 0.001.*

Regulation of rOSTα and rOSTβ in rat intestine and liver by the GR and PXR ligands. Incubation of rat intestinal (jejunum, ileum and colon) and liver slices with the GR/PXR ligand, DEX (1 μM and 50 μM for intestinal slices and 50 μM for liver slices), significantly induced the rOSTα and rOSTβ expression in jejunum, colon and liver (Figs. 7A, C and D) but not in the ileum (Fig. 7B). These results in the intestinal slices were displayed again with BUD (10 nM), the specific GR ligand, which induced rOSTα and rOSTβ expression in rat jejunum (Fig. 7A) and colon (Fig. 7C) but not in the ileum (Fig. 7B). However, the PXR ligand, PCN (10 μM), did not influence the rOSTα and rOSTβ expression in all regions of the intestine and liver slices (Figs. 7A, B, C and D). In contrast to DEX, neither PCN nor BUD (10 nM and 100 nM) induced rOSTα and rOSTβ expression in liver slices during 8 h of incubation (data not shown), whereas BUD (100 nM) significantly induced rOSTα expression (fold induction 2.9; $P < 0.05$) (Fig. 7D) during 24 h of incubation. Further, to confirm the intactness of the GR and PXR response in the rat intestinal (jejunum, ileum and colon) and liver slices, PXR, CYP3A1 and CYP3A9 mRNA expression were analyzed in these samples. The GR ligands, BUD and DEX, significantly induced PXR and CYP3A9 mRNA in all the segments of the intestine and in liver slices (13). The PXR ligands, PCN and DEX, induced CYP3A1 and CYP3A9 in a region specific manner in rat intestine and liver slices (13). This observation confirmed the intactness of the GR and PXR nuclear receptor pathways in the rat intestinal and liver slices.

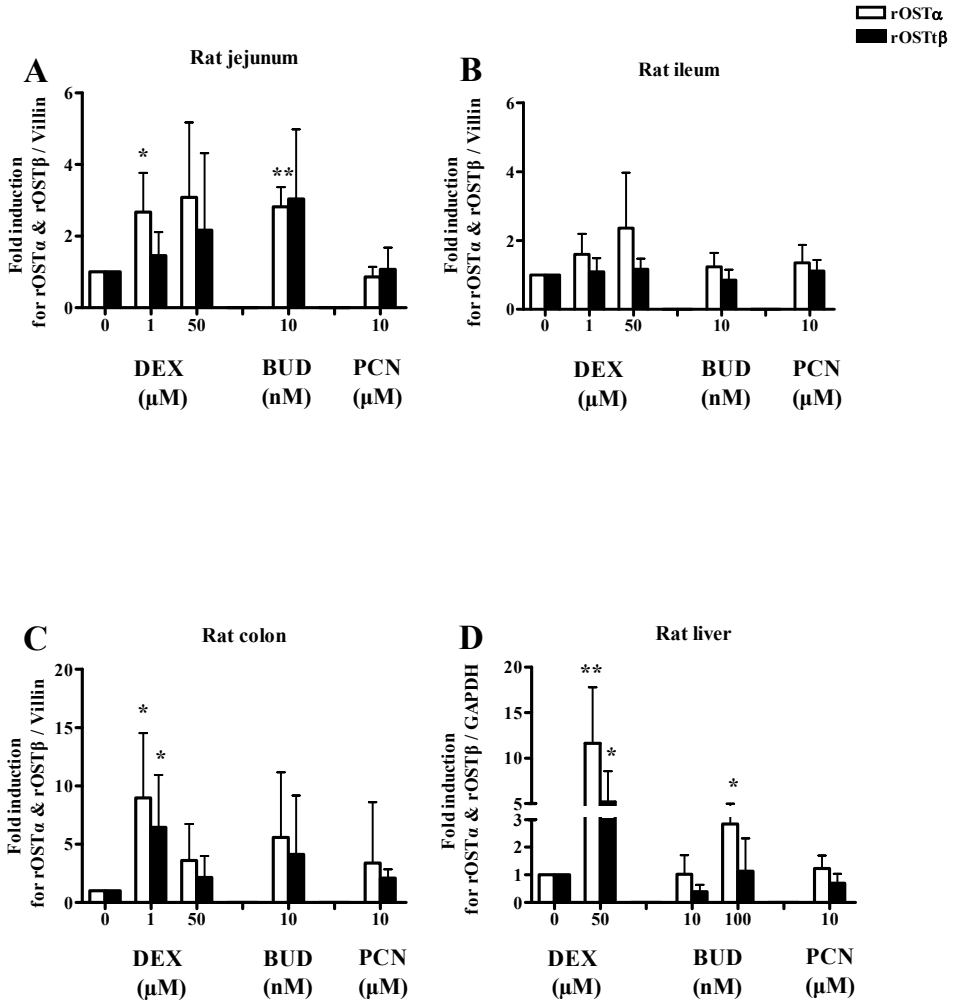

Figure 7. The GR ligands, dexamethasone (DEX) and budesonide (BUD) but not the PXR ligand, pregnane 16-α carbonitrile (PCN) induce the expression of rat rOSTα and rOSTβ genes in jejunum (A), colon (C), but not in ileum (B) and liver (D) slices. Rat jejunum, ileum and colon slices were treated with 1 μM and 50 μM of DEX, 10 nM of BUD, and 10 μM of PCN for 12 h. Rat liver slices were treated with 50 μM of DEX, 10 nM and 100 nM of BUD, and 10 μM of PCN for 24 h. Results are expressed as mean ± S.D. of 4-5 rats. "" indicates P < 0.05; and "**" indicates P < 0.001.*

Expression and regulation of hOSTα, hOSTβ in the human ileum and liver. The mRNA expression of hOSTα and hOSTβ relative to GAPDH in the ileum was 2- to 3-fold higher than that in human liver, which showed a low expression. The expression of hOSTα and hOSTβ mRNA in ileum slices was induced or remained unaltered upon incubation, with or without the solvents for 24 h (average C_T at 0 h, 26.0 for both hOSTα and hOSTβ and at 24 h, 25.0 for hOSTα and 24.0 for hOSTβ) (Fig. 8A). CDCA induced hOSTα in ileum slices 6- to 7-fold, but the effect on hOSTβ expression was smaller, only a 2.5-fold induction was

observed, and was consistently observed in all the human ileum donors (Fig. 8B). LCA, but not $1,25(OH)_2D_3$ moderately induced the hOSTα and hOSTβ expression in each of the 5 human ileum slice experiments (2- to 3-fold induction), but the levels failed to reach statistical significance due to the larger variation existing among the human ileum donor samples (Figs. 8B and C). DEX and BUD induced hOSTα and hOSTβ expression in all but one of the human ileum donors (Fig. 8D). CYP3A4 expression, a VDR, PXR, and GR responsive gene was significantly induced in these samples by $1,25(OH)_2D_3$, LCA, DEX and BUD but not by CDCA (13). Furthermore, CDCA and LCA significantly induced SHP expression (unpublished observations), confirming the intactness of the PXR, VDR, GR and FXR pathways in the ileum slices.

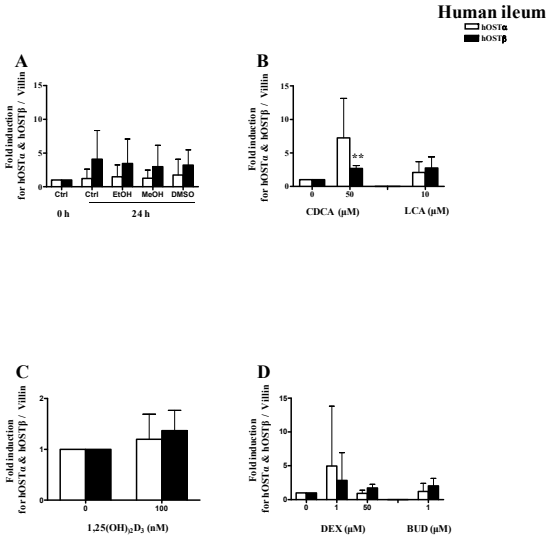

Figure 8. The effect of incubation time (24 h) and the solvent controls on the expression of human organic solute transporter, hOSTα and hOSTβ genes in human ileum slices (A). The mRNA expression of hOST α and hOST β genes relative to villin was quantified by real-time PCR and expressed with respect to the control slices before incubation, which were set to unity. The FXR ligands, CDCA and LCA, induced (B), the VDR ligand, $1,25(OH)_2D_3$ (C) did not induce, the GR/PXR ligand, DEX and the GR ligand, BUD (D) induced hOSTα and hOSTβ gene expression in all the experiments but failed to reach statistical significance. Results are expressed as mean ± SD of 4 to 5 human ileum donors ± S.D. "" indicates $P < 0.05$; and "**" indicates $P < 0.001$.*

The incubation conditions significantly decreased the expression (2-fold) of the hOSTα in human liver slices (C_T at 0 h, 27.0 and at 24 h, 28.0), whereas hOSTβ expression was significantly elevated (2-fold) (C_T at 0 h, 32.0 and at 24 h, 31.0) (Fig. 9A); these changes were not affected by the type of solvent used. Incubation of human liver slices with CDCA strongly induced hOSTα (15-fold induction) and hOSTβ (110-fold induction) expression. LCA moderately induced hOSTα and hOSTβ expression (2.5-fold and 3.5-fold respectively; $P < 0.05$) (Fig. 9B). $1,25(OH)_2D_3$ exerted only a minor decrease in hOSTα and hOSTβ expression in 3 out of 4 livers (Fig. 9C). DEX significantly decreased hOSTα expression and induced hOSTβ expression in 3 out of 5 livers (Fig. 9D). The intactness of the VDR, PXR, FXR and GR pathways in the slices was confirmed by increased CYP3A4 (13), SHP and PXR expression (unpublished observations).

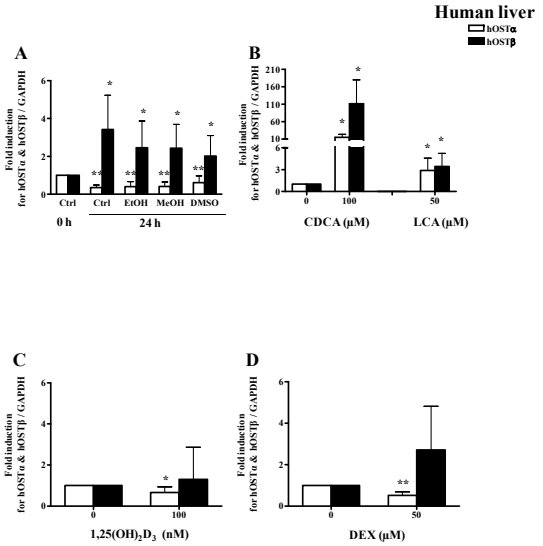

Figure 9. The effect of incubation time (24 h) and the solvent controls on the expression of human organic solute transporter (hOST)α and hOSTβ genes in human liver slices (A). The mRNA expression of hOSTα and hOSTβ genes relative to GAPDH were quantified by real-time PCR and expressed with respect to solvent treated controls, which were set to unity. The FXR ligands, CDCA and LCA induced (B) and the VDR ligand, 1,25(OH)$_2$D$_3$ (C) did not affect the expression hOSTα and hOSTβ genes. The GR/PXR ligand, DEX (D) significantly decreased the expression of hOSTα, but induced hOSTβ gene in 3 out of 5 livers. Results are expressed as mean ± SD of 4 or 5 human liver donors' ± SD. "" indicates P < 0.05; and "**" indicates P < 0.001.*

Discussion

In this study, precision-cut intact tissue slices of rat intestine (jejunum, ileum and colon) and liver, and human ileum and liver were used to investigate the species, organ and region dependent regulation of the basolateral bile acid half transporters, OSTα and OSTβ by FXR, VDR, PXR and GR ligands at the level of mRNA and the data are summarized in table 2. As shown by both qRT-PCR and immunohistochemistry (Figs. 1 and 2), rOSTα and rOSTβ were expressed in all regions of the rat small intestine and colon of Wistar rats, with the highest expression in ileum, where most of the bile acids are actively reabsorbed (33). Although the absolute expression of these genes cannot be determined by the applied qRT-PCR technique, the relative expression of these genes along the length of the intestine can be assessed. The expression patterns of rOSTα and rOSTβ in the rat intestine paralleled that of rASBT as reported earlier in mouse (7), the Sprague Dawley rat and man (1), and their concomitant presence supports the hypothesis that they are both involved in the facilitation of bile acid absorption. The ratio of their expression in ileum relative to that in jejunum was higher in Wistar rats (3.8-fold for OSTα and 8-fold for OSTβ, when normalized for villin expression) (Fig. 1) than the 2-fold difference reported for Sprague Dawley rats (1). The rOSTα and rOSTβ are expressed at the basolateral surface of the ileal enterocyte with a decreasing gradient of expression from the villus tip to the crypts in the Wistar rats, which is similar to the earlier reports in the mouse and the Sprague Dawley rats (1, 34). In the livers of Wistar rats, rOSTα and rOSTβ proteins were found to be expressed in detectable intensities at the basolateral membranes of the BEC (Fig. 2). Furthermore, rOSTα and rOSTβ proteins were also detected, albeit at lower intensities, at the basolateral membranes of the hepatocytes, as documented in earlier reports (1). However their functional significance in hepatocytes has not been proven to date.

Previously, we showed that both rat and human intestinal and liver slices adequately reflect the regulation of drug metabolising enzymes and transporters as observed *in vivo* (25, 38), and VDR, PXR, GR and FXR pathways remained intact (13). The quality of total RNA isolated from fresh tissue (rat and human) and the 0 h slices prior to incubation was similar and no change was observed during the period of cold storage and slicing in the average C_T for rOSTα, rOSTβ, rVDR, rFXR, and rPXR genes, as well as the signature genes of the various nuclear receptors. Viability (ATP levels) and housekeeping genes (villin and GAPDH) remained constant during the incubation (data not shown). The expression of rOSTα and rOSTβ mRNA were moderately altered during incubation of rat intestinal and liver slices. In rat jejunum, the expression of rOSTβ was significantly elevated after 12 h of incubation. In contrast, rat liver slices showed a significant decrease in the expression of rOSTβ without affecting rOSTα expression. However, in rat ileum, rOSTα and rOSTβ expression was significantly increased and in rat colon, rOSTα and rOSTβ expression was significantly decreased. These changes in rOSTα and rOSTβ expression in different segments of the rat intestine and liver during incubation of the slices suggest that the expression of the rOSTα and rOSTβ genes is normally regulated *in vivo* by endogenous factors which seem to be absent in the culture medium or by endogenously generated factors whose availability is altered during incubation of the slices.

As reported earlier (3, 9, 15), CDCA, a high affinity FXR ligand, was found to induce OSTα and OSTβ genes in rat and human ileum and liver slices (Figs. 4B, 4D, 8B and 9B). The induction of hOSTα and hOSTβ genes in human ileum (6- to 7-fold) and liver (15- and 110-fold) slices by CDCA was much stronger than that reported earlier in human ileum biopsies incubated for 4 h only, and in human hepatoma cell lines, Huh 7 and HepG2 (15). Our results show that the rat rOSTα and rOSTβ genes, similar to human and mouse OSTα and OSTβ genes (9, 15) are responsive to CDCA in intact cells.

The presence of detectable amounts of rOSTα and rOSTβ mRNA and protein (Figs. 1 and 2) and rFXR mRNA not only in the ileum but also in the jejunum and colon of the rat intestine (13), and the reported induction of *Ostα and Ostβ* genes by CDCA in ceacum and colon of Slc10a2$^{-/-}$ mouse by Frankenberg et.al. (9) prompted us to investigate the regulation of rat rOSTα and rOSTβ genes in the rat jejunum and colon by the FXR ligand, CDCA. Similar to what was observed for the rat ileum, the rOSTα and rOSTβ genes were also significantly induced by CDCA in rat jejunum and colon (Figs. 4A and C). This shows that, although the basal expression pattern of rOSTα and rOSTβ genes varied widely along the length of the rat intestine, the half transporters were responsive to the FXR stimulus, albeit to a different extent in all regions of the intestine. These results also show that bile salts, despite being present at high concentrations in both jejunum and ileum lumen *in vivo*, do not play a decisive role in the basal expression of rOSTα and rOSTβ in the small intestine. In the rat colon, the response of the rOSTα and rOSTβ promoters to CDCA appeared to be remarkably higher than in ileum, while the expression of FXR in colon is similar to ileum (13). Based on these results, we speculate that bile acids play a role in the

regulation of their own absorption by increasing their basolateral excretion in the intestine. The difference in the CDCA-induced response of rOSTα and rOSTβ between ileum and colon might be due to a higher intracellular concentration of CDCA in the colon which might be the result of a different balance between uptake, excretion and/or metabolism.

Further, the role of the nuclear receptors, GR and VDR in the regulation of rOSTα and rOSTβ genes is investigated by incubating rat jejunum, ileum, colon and liver, human ileum and liver slices with GR and VDR ligands. DEX was found to significantly induce rOSTα and rOSTβ expression in rat jejunum, colon and liver, but the moderate induction of rOSTα and rOSTβ expression in rat ileum slices was found to be not significant (Figs. 7A, B, C and D). In addition, in human ileum slices, both hOSTα and hOSTβ gene expression was induced by DEX (Fig. 8D). In human liver slices, DEX significantly decreased the expression of hOSTα but induced hOSTβ expression (Fig. 9D). These results are the first to show that rat and human OSTα and OSTβ genes are regulated by DEX in intestine and liver. The induction of OSTα and OSTβ genes by DEX in rat intestine and liver, and human ileum is likely to be attributed to GR and not to PXR, since BUD, a specific GR ligand, also induced the expression of OSTα and OSTβ genes. The PXR ligand, PCN, failed to alter rOSTα and rOSTβ in rat intestinal slices. Furthermore GR ligands induce the trans-acting factor, LRH-1 (chapter 5), which is reported to be essential for the basal expression of OSTα and OSTβ (17). However, whether the effects of the GR ligands are indirectly mediated through induction of HNF4α and LRH-1, or directly mediated through a potential GRE in the OSTα and OSTβ genes needs to be ascertained. These results on the induction of OSTα and OSTβ by GR ligands further explain the decreased loss of bile acids in the feaces and increased bile acid absorption in the patients with crohn's disease treated with BUD and DEX. It was reported that these patients have induced ASBT expression (12). Furthermore, induction of ASBT was found in human ileum slices treated with GR ligands (chapter 5) with subsequent induction of OSTα and OSTβ (Fig. 8D). Thus, GR ligands simultaneously increase ASBT, OSTα and OSTβ expression in human ileum slices.

In addition, our data also provide evidence on the involvement of the VDR in the regulation of rat rOSTα and rOSTβ genes. $1,25(OH)_2D_3$ exerted an inhibitory effect on the expression of rOSTα and rOSTβ genes in rat jejunum, ileum and colon slices in a dose-dependent manner (Figs. 5A, B and C) but had no effect in liver slices (Fig. 5D). In the human ileum, hOSTα and hOSTβ genes were not significantly altered by $1,25(OH)_2D_3$ treatment, whereas in the human liver slices, hOSTα expression was significantly decreased in all the 4 livers, and 3 out of 4 livers exhibited a 50% decrease in the expression of hOSTβ (Fig. 9C). Hence, the role of VDR on the regulation of the OST genes is different for hOSTα and hOSTβ genes in humans and appeared to differ among tissues and in different species. The involvement of the VDR was further investigated with another natural VDR ligand, LCA with affinity towards FXR (20, 27). LCA was found to decrease the expression of rOSTα and rOSTβ genes significantly in rat ileum (Fig. 4B). However, in rat liver, LCA induced rOSTα expression without affecting the rOSTβ expression (Fig.

4D). This inductive effect of LCA on rOSTα in rat liver was in contrast to that of $1,25(OH)_2D_3$, but paralleled that of CDCA suggesting that LCA acted as a FXR ligand (27). However, LCA showed opposite results in rat jejunum and colon. In the rat jejunum, LCA significantly down regulated rOSTα without affecting rOSTβ, whereas in rat colon, LCA significantly induced rOSTβ without affecting the rOSTα expression. These mixed results suggest that LCA affects the expression of rOSTα and rOSTβ genes via both VDR and FXR, giving rise to inhibition and induction, respectively. The different effects of LCA on the rOSTα and rOSTβ genes in rat intestine and liver are difficult to interpret but suggest that the FXR-mediated effects predominate in rat colon and liver, whereas the VDR-mediated effects predominate in jejunum and ileum. For the rat liver, this may be explained by the higher expression of FXR compared to VDR in comparison with the intestine. (13). In human liver slices, LCA significantly induced the hOSTα and hOSTβ expression, similar to that of CDCA (Fig. 9B), however LCA induced hOSTα and hOSTβ expression in all the human ileum donors but failed to reach statistical significance (Fig. 8B), suggesting that the FXR regulation predominates in humans, which is in line with the lack of VDR-mediated effect by $1,25(OH)_2D_3$ (Fig. 8C). The results on induction of hOSTα and hOSTβ by LCA and CDCA in human livers are in line with those reported by Zollner et al (44) in human cholestatic livers. Based on these results, it might be speculated that during cholestasis, bile acids might play a role in the rescue phenomenon by inducing the OSTα/OSTβ transporter present in the basolateral membranes of human hepatocytes as **w**as also suggested for MRP3 (37). Together OSTα/OSTβ and MRP3 play a protective role by increasing the bile acid efflux into the blood from the hepatocytes. Furthermore, the different effects of LCA on the rOSTα versus the rOSTβ expression in rat jejunum, ileum and colon, and in human liver are noteworthy because it is reported that the functional bile acid basolateral transporter is a heterodimer of OSTα and OSTβ proteins (19, 36, 41). These results necessitate further studies to investigate the effect of LCA on the formation of the functional OSTα-OSTβ transporter.

To mimic the *in vivo* situation where $1,25(OH)_2D_3$ was shown to increase the flux of bile acids into the rat ileocytes by inducing rASBT (5), rat ileum slices were co-incubated with both $1,25(OH)_2D_3$ and CDCA. $1,25(OH)_2D_3$ completely abolished the CDCA-mediated induction of rOSTα and rOSTβ (Fig. 6A) despite the presence of an intact FXR response, shown by the induced rSHP expression (Fig. 6B). Altogether, these results led to the postulate of a negative VDRE in the promoters of the rat rOSTα and rOSTβ genes, as reported earlier for the parathyroid and CYP7A1 genes (10, 32), that overrides the FXR-dependent positive regulation of rOSTα and rOSTβ genes by CDCA. However, indirect effects of $1,25(OH)_2D_3$ on the expression of rOSTα and rOSTβ genes cannot be ruled out. Further studies are needed to ascertain this hypothesis.

In conclusion, this study showed the induction of hOSTα and hOSTβ genes by the FXR ligand, CDCA, in intact human ileum and liver tissue, and confirmed the earlier reports of human ileum biopsies and HepG2 cells (15). Induction of rOSTα and rOSTβ

gene expression by CDCA in rat jejunum, ileum, colon and liver suggests that the rOSTα and rOSTβ promoters are responsive to FXR ligand, observations that are similar to the mouse and human. Furthermore, the rat but not human OSTα and OSTβ genes are negatively regulated by the VDR ligand, $1,25(OH)_2D_3$. This data suggests that the toxic bile salt, LCA acts as a VDR ligand on rat rOSTα and rOSTβ genes rather than as an FXR-ligand in jejunum and ileum, but acts as an FXR-ligand in the rat colon and liver, and in human ileum and liver. This study reports here, for the first time, that the rat and human OSTα and OSTβ genes are not only positively regulated by FXR, but also by GR ligands. In conclusion, apart from FXR, also VDR and GR ligands, which were implicated in the regulation of ASBT expression in rat and human intestine and liver, regulate the mRNA expression of the OSTα and OSTβ genes, as summarized in table 2. However, the changes in expression of these two half transporters is often not identical and the physiological consequences remains to be elucidated.

Table 2 Summary of the effects of VDR, FXR, PXR and GR ligands on the OSTα and OSTβ expression in rat and human intestine and liver; n=4-5 rats or 3-5 human ileum and liver donors.

Ligand(s)	Nuclear receptor	Rat								Human			
		Intestine						Liver		Ileum		Liver	
		Je		Il		Co							
		rOSTα	rOSTβ	rOSTα	rOSTβ	rOSTα	rOSTβ	rOSTα	rOSTβ	hOSTα	hOSTβ	hOSTα	hOSTβ
$1,25(OH)_2D_3$	VDR	↓	↓	↓	↓	↓	↓	↔	↔	↔	↔	↓	↔
CDCA	FXR / VDR	↑	↑	↑	↑	↑	↑	↑	[a]↑	↑	↑	↑	↑
LCA	FXR / VDR	↓	↔	↓	↓	↔	↑	↑	[a]↑	↑	↑	↑	↑
DEX	PXR / GR	[a]↑	[a]↑	[a]↑	↔	[a]↑	[a]↑	↑	[a]↑	↔	[a]↑	↓	[b]↑
PCN	PXR	↔	↔	↔	↔	↔	↔	↔	↔	ND		ND	
BUD	GR	↑	[a]↑	↔	↔	[a]↑	[a]↑	↑	[a]↑	↔	[a]↑	ND	

J – Jejunum; IL – Ileum; Co – Colon
↑- induction; ↓ - repression; ↔ no induction
[a]↑- Induction in all experiments but with high variation between the experiments
[b]↑ - Induction in 3 out of 5 experiment
ND-Not done

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