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

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Chapter 5

Regulation of ASBT (SLC10A2) by VDR, FXR and GR ligands in Rat and Human Ileum and Liver

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Abstract:

The apical sodium dependent bile acid transporter (ASBT) plays an important role in the homeostasis of bile acids and cholesterol. However its regulation is not fully understood. In this study we investigated and compared the direct effects of ligands for nuclear receptors such as VDR, FXR and GR on the regulation of ASBT in rat and human ileum and liver using precision-cut tissue slices at the level of mRNA, and the changes in the mRNA profile of transcription factors regulating the ASBT expression such as SHP, HNF1α, HNF4α and LRH1 were also investigated. Further we compared the *in vitro* effect of VDR ligand, 1α,25dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) with the *in vivo* results obtained by treating the Wistar rats for 12 h and 4 days with $1,25(OH)_2D_3$ (1200 pmol/kg/day) intraperitoneally (ip). The VDR ligand, $1,25(OH)_2D_3$ significantly decreased ASBT expression in rat and human ileum, which can be explained by a simultaneous decrease in HNF1α expression. Furthermore $1,25(OH)_2D_3$ decreased ASBT expression in rat liver but not in human liver slices. The toxic bile acid, lithochochlic (LCA) decreased ASBT expression in rat and human ileum, whereas the FXR ligand chenodeoxycholic acid (CDCA) did not affect ASBT expression in rat ileum, but decreased ASBT expression in human ileum, possibly as a result of the concomitant induction of SHP. In rat and human liver slices, LCA did not affect ASBT expression, but CDCA induced ASBT expression in rat liver slices but not in human liver slices. As expected, the GR ligands, DEX induced ASBT expression in rat and human ileum slices with simultaneous induction of HNF1α, and also induced ASBT in rat and human liver slices. Treatment of Wistar rats *in vivo* with $1,25(OH)_2D_3$ did not result in altered ASBT expression in ileum and liver but induced it in the kidney.. The apparent discrepancy in the effects of $1,25(OH)_2D_3$ in *in vitro* and *in vivo* experiments might be attributed to differences in the exposure of rat ileum to effective concentration of $1,25(OH)_2D_3$.

Abbreviations: $1,25(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; VDR, vitamin D receptor; NR, nuclear receptor, C_T, comparative threshold cycle number.

Keywords: ASBT, regulation, nuclear receptors, intestinal slices, liver slices

Introduction

The apical sodium dependent bile acid transporter (ASBT; SLC10A2) is a bile acid transporter localized to the apical surface of the terminal ileal enterocytes, large cholangiocytes (BEC) and the renal proximal tubular cells (30, 45). ASBT plays an important role in the reabsorption of bile acids in the ileum and in the bile ducts in the enterohepatic cycle at the renal proximal tubules (13, 30). During the enterohepatic cycle, 95% of the BA's are reabsorbed in the terminal ileum and transported across the basolateral membranes by heterodimeric organic solute transporter, OSTα/OSTβ into the portal blood and transported to the liver (4, 14, 44, 52). In the liver, the hepatocytes takes up the bile acids by the sinusoidal sodium-dependent taurocholate co-transporting polypeptide (NTCP; SLC10A1) (16), and other sodium-independent organic anion transporting polypeptides, OATP1B1 (SLC01B1), OATP1A2 (SLC01A2) and OATP1B3 (SLC01B3) (2).

The basal expression of ileal ASBT is regulated at the transcription level, which plays an important role in maintaining the bile acid pool *in vivo* (8, 22, 23, 46) by at least three transcription factors, the hepatocyte nuclear factor 1α (HNF1α), activation protein (AP) 1, c-jun, and liver receptor homologue protein 1 (LRH-1) (9, 10, 36, 43). Furthermore, human and rat ASBT expression is positively regulated by glucocorticoid receptor (GR; NR3C1) ligands, as was shown *in vitro* and *in vivo* experiments (25, 38). *In vitro*, the human ASBT promoter is activated by the peroxisome proliferator activated receptor α (PPARα; NR1C1) and the rat ASBT promoter is activated by the vitamin D receptor (VDR; NR1I1) (11, 26). The ligand activated GR, PPARα and VDR bind to their respective response elements in the ASBT promoter as a homodimer or heterodimer with the retinoic acid X receptor α (RXRα; NR1B1) (26, 48) to induce ASBT expression. Furthermore, ASBT is negatively regulated in the ileum by the reabsorbed bile acids in mouse, guinea pigs and rabbits with effects mediated through the farnesoid X receptor (FXR; NR1H4) (32, 33, 37, 49). The bile acid-activated FXR forms a heterodimer with RXRα (retinoic acid X receptor α) and induces the expression of the short heterodimer protein (SHP; NR0B2), which in turn, down-regulates ASBT expression by inhibiting the activity of LRH-1 in mouse and humans (10). Also in Caco-2 cells, ASBT is down regulated by bile acids, and the decreased ASBT expression was found in human gallstone patients (6). However no data is available on the regulation of ASBT by bile acids directly in the human intestinal tissue. In the rat, the regulation of the ASBT gene by bile aicds is controversial with reports suggesting positive (21, 47) or negative regulation (17, 41), or no effect (3). Chen et al. (10) found that, in contrast to the mouse and the human ASBT promoter, the LRH-1 binding site is absent in the rat ASBT promoter. This explained the absence of negative feedback regulation of the rat intestinal ASBT by the bile acid-liganded FXR, in as much as the FXR-SHP-LRH1 cascade would not play a role in the regulation of rat intestinal ASBT (10).

Most of the data on ASBT regulation by bile acids in the rat liver are obtained from the cholestatic, rodent model, suggesting induction (1), repression (29) or no effect (3). In human livers, no data is available on the regulation of ASBT. Moreover, the ASBT regulation data obtained *in vivo* may not discriminate between the direct effects of the bile acids vs the indirect effects resulting from other changes induced in the intestine. Also, there is no data available on the direct effects of ligands for various nuclear receptors on the regulation of ASBT in the rat and human liver.

Therefore, we performed a systematic study to investigate the direct effects of VDR ($1,25(OH)_2D_3$ and LCA), FXR (CDCA and GW4064) and GR (dexamethasone (DEX) and budesnoide (BUD)) ligands on the regulation of ASBT mRNA expression in rat and human intestine and liver using precision-cut tissue slices This *ex-vivo* model was previously shown to be an adequate model to study the regulation of genes of interest by ligands for various NR in the rat and human intestine and liver under identical experimental conditions (20, 24, 27, 28, 35, 39, 50). Furthermore, we also analyzed the effects of VDR, FXR and GR ligands on the NR/transcription factors such as SHP, HNF1α, HNF4α and LRH1, which were reported to be involved in the regulation of ASBT expression (19). Subsequently, we compared the *in vitro* effect of $1,25(OH)_2D_3$ on the regulation of ASBT in rat ileum and liver slices with those obtained *in vivo* by intraperitoneal treatment (ip) of Wistar rats with $1,25(OH)_2D_3$, and also analyzed total bile acid concentrations in the portal and systemic blood.

Materials and methods

Animal and Human Tissue. Male Wistar (HsdCpb:WU) rats weighing about 230 - 250 g were purchased from Harlan (Horst, The Netherlands). Pieces of human liver tissue were obtained from patients undergoing partial hepatectomy for the removal of carcinoma or from redundant parts of donor livers remaining after split liver transplantation as described previously by Olinga et al. (40), and human liver donor characteristics are as reported earlier (27). 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. The experimental protocols involving animals and human tissue were approved by the Animal Ethical Committee of the University of Groningen and Medical Ethical Committee of the University Medical Center, Groningen, respectively.

$1,25(OH)_2D_3$ in ethanol was purchased from BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA. Chenodeoxycholic acid (CDCA) and lithocholic acid (LCA) was purchased from Calbiochem, San Diego, CA, USA. Dexamethasone (DEX) was purchased from Genfarma bv, Maarssen. Ethanol and methanol were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO); Gentamicin and Williams medium E with glutamax-I and amphotericin B (Fungizone)-solution were obtained from Gibco (Paisley, UK). GW4064 was purchased from Tocris Bioscience (Bristol, UK). Polymethyl sulfonyl

flouride (PMSF), and dithiothreitol (DTT) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). D-Glucose and HEPES were procured from ICN Biomedicals, Inc. (Eschwege, Germany). Low-gelling temperature agarose and budesonide (BUD) was purchased from Sigma–Aldrich (St. Louis, MO). Total bile acid assay kit was purchased from Diazyme laboratories, San Diego, CA, USA. RNAeasy mini columns were obtained from Qiagen, Hilden, Germany. Random primers (500 μg / ml), $MgCl_2$ (25 mM), RT buffer (10x), PCR nucleotide mix (10 mM), AMV RT (22 U/μl) and RNasin (40 U/μl) were procured from Promega Corporation, Madison WI, USA. SYBR Green and Taq Master Mixes were purchased from Applied Biosystems, Warrington, UK and Eurogentech, respectively. All primers were purchased from Sigma Genosys. All reagents and materials used were of the highest purity that was commercially available.

Preparation of rat and human intestinal and liver slices. The rat ileum and liver were excised under isoflurane/O_2 anaesthesia. The ileum was 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. After surgical resection, the human ileum tissue was immediately placed in ice-cold carbogenated KHB and human liver was placed in ice-cold UW. Human liver and ileum precision-cut slices were prepared within 30 to 60 min after resection according to the earlier published methods (15, 27, 28, 40, 51).

Induction studies. Rat and human precision-cut ileum slices 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 (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. Rat and human ileum slices were incubated with $1,25(OH)_2D_3$ (5-100 nM), CDCA (50 μM), LCA (5-10 μM) and DEX (1-50 μM). Rat ileum slices were incubated with GW4064 (1 μM) and human ileum slices were incubated with BUD (1 μM). Rat ileum slices were incubated for 8 h and 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, since villin expression was stable up to 24 h. From a single rat or single human ileum donor, six or 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 quantitative real time PCR (qRT-PCR) analysis. Samples were stored in – 80° C freezer until RNA isolation. These experiments were replicated in 3 to 5 rats and 3 to 5 human ileum donors.

Rat and human liver slices 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 humidified carbogen, kept at 37° C and continuously gassed with carbogen, and shaken at 80 rpm. Rat and human liver slices were incubated with 1,25$(OH)_2D_3$ (10-200 nM), CDCA (10-100 μM), LCA (10-50 μM) and DEX (1-50 μM). Rat liver slices were incubated with GW4064 (1 μM). Control slices were incubated in supplemented Williams medium E with 1% ethanol or 1% methanol or 1% DMSO without inducers. Rat and human liver slices were incubated for 8 h and 24 h, respectively. From a single rat or 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 in – 80° C freezer until RNA isolation. These experiments were replicated in 3 to 5 rats and 4 to 5 human liver donors.

In vivo studies. Wistar rats were divided into two groups with twelve animals each. In both groups six animals were treated with 1,25 $(OH)_2D_3$ and six served as controls. Treated animals received 1200 pmol/kg/day 1,25 $(OH)_2D_3$ in corn oil by intraperitoneal injection (ip) and corresponding controls received the same volume of corn oil. Group I animals were sacrificed at 12 h after the first dose. Group II animals were treated once daily for four days and sacrificed 24 h after the last dose. The small intestines (jejunum and ileum), colon, livers and kidneys were collected in ice-cold phosphate buffered saline (PBS) containing the protease inhibitors PMSF (1 mM) and DTT (0.5 mM). The intestinal segments were flushed with ice-cold PBS with PMS and DTT, and divided into small pieces and snap-frozen in liquid nitrogen. Pieces of liver and kidney were also snap-frozen in liquid nitrogen. Samples were stored at – 80° C until RNA isolation.

RNA isolation and qRT-PCR. Total RNA was isolated from rat and human intestine and liver samples 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 in all the samples. About 2 μg of total RNA in 50 μl was reverse-transcribed into template cDNA as reported earlier (27).

qRT-PCR for the rat and human genes of interest was performed either by SYBR Green or Taqman® analysis according to the availability of primer sets; villin and GAPDH were used as house-keeping genes for intestinal and liver samples, respectively. Rat and human ASBT mRNA was analysed by Taqman® analysis. Rat ASBT Taqman primers and probes are forward primer (5'-3') ACCACTTGCTCCACACTGCTT, reverse primer (5'-3') CGTTCCTGAGTCAACCCACAT and probe (5'-3') FAM-CTTGGAATGATGCCCCTTTGCCTCT-TAMRA; human ASBT Taqman primers and probes are forward primer (5'-3') ACGCAGCTATGTTCCACCATC, reverse primer (5'-3') GCGGGAAGGTGAATACGACA and probe (5'-3') FAM-TTCAGCTCTCCTTCACTCCTGAGGAGCTC-TAMRA; β-actin expression was analyzed

by Taqman using assay by design primer sets obtained from Applied Biosystems, Warrington, UK. Human villin and GAPDH SYBR Green primer sequences were similar to those reported earlier by us (27). Rat and human SHP, HNF1α and HNF4α, and rat LRH1 was analyzed by SYBR green primer sets, as given in chapter 3. 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 analysis, ~ 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 (27). All samples were analyzed in duplicates in 384 well plates using ABI7900HT from Applied Biosystems. Appropriate controls were analyzed to detect potential primer dimer formation and DNA contamination in the samples. Dissociation curves showed a single homogenous product for all the primer sets. The comparative threshold cycle (C_T) method 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 or β-actin for intestine and GAPDH for liver) ($^{\Delta}C_T$), which was compared to the corresponding $^{\Delta}C_T$ 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 $2^{-(\Delta\Delta CT)}$.

Bile acid estimation in portal and systemic blood. Bile acid concentrations was determined in portal and systemic blood of control and $1,25(OH)_2D_3$ treated rats using a colorimetric total bile acids assay kit from Diazyme laboratories, San Diego, CA, USA by following the manufacturer's protocol.

Statistical analysis. All values were expressed as the mean ± S.E.M. All data were analyzed by paired student's *t*-test using SPSS Version 16 for significant differences between/among the means of treatment. Statistical analysis was performed on fold induction as well as on $^{\Delta\Delta}C_T$ with similar results. The *P* value < 0.05 was considered as significant.

Results

Regulation of ASBT expression in rat ileum and liver slices. The ASBT mRNA was significantly decreased during control incubations of rat ileum slices for 12 h and 24 h (Fig. 1A), but was not influenced by the solvent (data not shown). Incubation of rat ileum slices with $1,25(OH)_2D_3$ significantly decreased the ASBT expression (60 % expression at 100 nM $1,25(OH)_2D_3$ vs. control incubated slices; $P < 0.05$) (Fig. 1B). Among the bile acids, CDCA did not affect ASBT expression in rat ileum slices (Fig. 2A). LCA significantly decreased ASBT expression in rat ileum slices (40 % expression at 10 µM LCA vs. control incubated slices; $P < 0.05$) (Fig. 2A). Co-incubation of rat ileum slices with $1,25(OH)_2D_3$ or LCA and CDCA decreased the ASBT expression (40 % expression at 100 nM

$1,25(OH)_2D_3$ or 10 μM LCA vs. control incubated slices; $P < 0.05$) (Fig. 2B). The FXR ligand, GW4064 did not affect ASBT expression in rat ileum slices, whereas DEX significantly induced ASBT expression (2-fold induction; $P < 0.05$) (Fig. 2A).

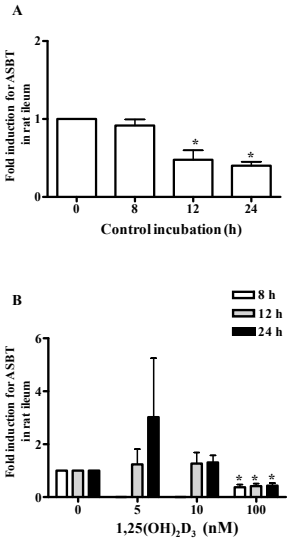

*Figure 1. Rat ileum slices were exposed to incubation with vehicle (A), and $1,25(OH)_2D_3$ (5-100 nM) (B) for 8 h, 12 h and 24 h, after which total RNA was isolated and mRNA expression of ASBT was evaluated by qRT-PCR. Results were expressed as fold-induction after normalizing with β-actin, and compared with the control incubated slices for the same length of time, which was set to unity. The results showed mean ± S.E.M. of 3-5 rats; in each experiment 6 ileum slices were incubated per condition. Significant differences towards the control incubations are indicated with * for $P < 0.05$.*

In rat liver slices, ASBT mRNA expression was not affected during 8 h and 24 h control incubation (Fig. 3A) and not influenced by the solvents (data not shown). $1,25(OH)_2D_3$ decreased ASBT expression (Figs. 3B and C). CDCA and LCA significantly induced ASBT expression relative to their respective solvent incubated controls (Figs. 3B and C), whereas GW4064 did not affect ASBT expression (Figs. 3B and C). DEX induced ASBT expression in liver slices (Fig. 3B).

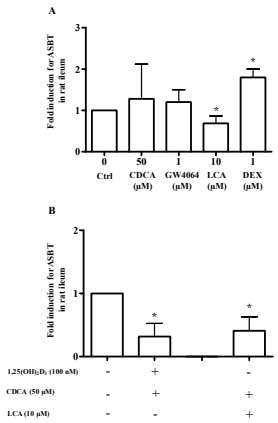

*Figure 2. Slices from rat ileum were exposed to CDCA (50 μM), LCA (5-10 μM), GW4064 (1 μM) and DEX (1 μM) for 8 h (A), $1,25(OH)_2D_3$ (100 nM) + 50 μM of CDCA and LC (10 μM) + 50 μM of CDCA for 8 h (B) after which total RNA was isolated and mRNA expression of ASBT was evaluated by qRT-PCR. Results were expressed as fold-induction after normalizing with β-actin, and compared with the control incubated slices for the same length of time, which was set to unity. Results showed mean ± S.E.M. of 3-5 rats; in each experiment 6 ileum slices were incubated per condition. Significant differences towards the control incubations are indicated by * for $P < 0.05$.*

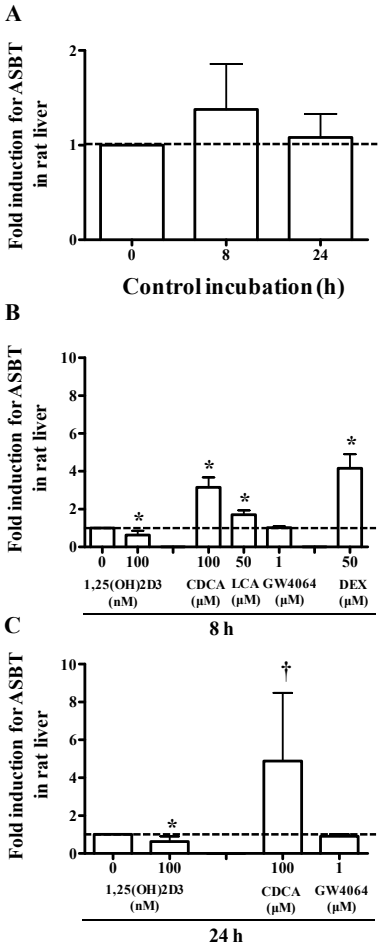

*Figure 3. Rat liver slices were exposed to incubation with vehicle for 0 h, 8 h and 24 h (A), 1,25(OH)$_2$D$_3$ (100 nM), CDCA (50 μM), LCA (50 μM), GW4064 (1 μM) and DEX (50 μM) for 8 h (B) and 1,25(OH)$_2$D$_3$ (100 nM), CDCA (50 μM) and GW4064 (1 μM) for 24 h (C), after which total RNA was isolated and mRNA expression of ASBT was evaluated by qRT-PCR. Results were expressed as fold-induction after normalizing with β-actin, and compared with the control incubated slices for the same length of time, which was set to unity. The results showed mean ± S.E.M. of 3-5 rats; in each experiment 3 liver slices were incubated per condition. Significant differences towards the control incubations are indicated by * for P < 0.05.*

Regulation of SHP, HNF1α, HNF4α and LRH-1 expression in rat ileum and liver slices. The expression of SHP, HNF1α, HNF4α and LRH-1 mRNA was found to decrease during control incubations of rat ileum slices but was not influenced by the solvents during 8 h and 12 h incubation (data not shown). Incubation of rat ileum slices with 1,25(OH)$_2$D$_3$ for 8 h and 12 h did not affect SHP, HNF4α and LRH-1 expression (data shown for 12 h only) (Fig. 4), whereas HNF1α expression was significantly decreased (50 % expression at 100 nM 1,25(OH)$_2$D$_3$ vs control incubated slices; $P < 0.05$) (Fig.4). CDCA and LCA induced SHP expression (2-fold induction; $P < 0.05$) (Fig.4). CDCA induced HNF1α and HNF4α expression but not LRH-1 expression (Fig. 4), whereas LCA decreased HNF1α expression without affecting HNF4α and LRH-1(Fig. 4). DEX induced SHP, HNF1α and LRH-1 but not HNF4α mRNA expression in rat ileum slices (Fig. 4). Co-incubation of rat ileum slices with 1,25(OH)$_2$D$_3$ and CDCA induced SHP expression (2-fold induction; $P < 0.05$) (28).

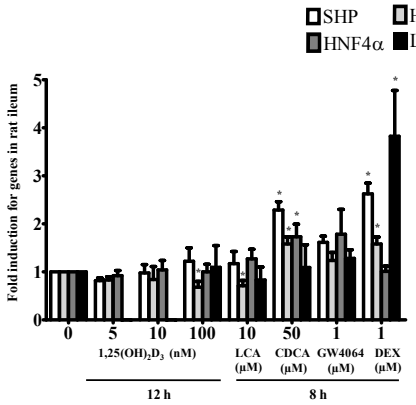

*Figure 4. Slices from rat ileum were exposed to 1,25(OH)$_2$D$_3$ (5-100 nM), CDCA (50 μM), LCA (10 μM), GW4064 (1 μM) and DEX (1 μM) for 8 h or 12 h after which total RNA was isolated and mRNA expression of SHP, HNF1α, HNF4α and LRH-1 was evaluated by qRT-PCR. Results were expressed as fold-induction after normalizing with villin, and compared with the control incubated slices for the same length of time, which was set to unity. The results showed mean ± S.E.M. of 3-5 rats; in each experiment 6 ileum slices were incubated per condition. Significant differences towards the control incubations are indicated by * for P < 0.05.*

Regulation of ASBT in rat ileum, liver and kidneys – In vivo. The administration of 1,25(OH)$_2$D$_3$ to Wistar rats at 1200 pmol/kg/day for 12 h and 4 days by ip injection did not affect ASBT mRNA in ileum and liver but ASBT expression was significantly induced in kidneys (2-fold induction; $P < 0.05$) (Figs. 5A and B).

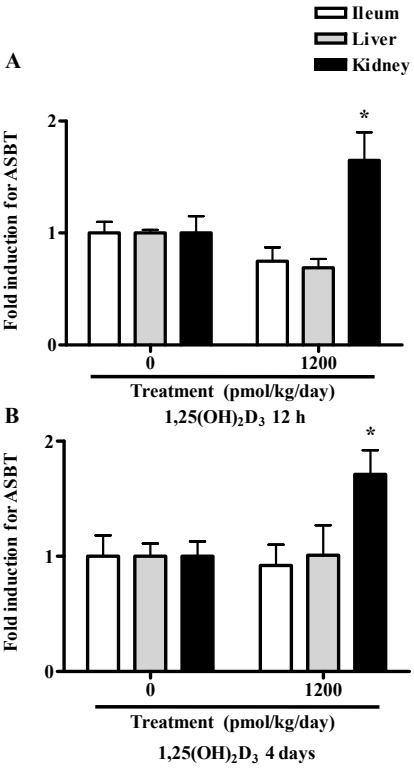

*Figure 5. Wistar rats were treated with with 1,25(OH)$_2$D$_3$ (1200 pmol/kg/day) for 12 h (A) and 4 days (B) by intraperitoneal injection. After 12 h or 24 h of the last dose administered, rats were sacrificed and total RNA was isolated from ileum, liver and kidneys and mRNA expression of ASBT in ileum, liver and kidney was evaluated by qRT-PCR. The results were expressed as fold-induction after normalizing with β-actin and compared with the control rats treated with vehicle for the same duration of treatment.. Results showed mean ± S.E.M. of 3 to 6 rats. Significant differences towards the vehicle treated rats were indicated by * for P < 0.05.*

Regulation of SHP, HNF1α, HNF4α and LRH-1 expression in rat ileum and liver – In vivo. The administration of $1,25(OH)_2D_3$ to Wistar rats at 1200 pmol/kg/day for 12 h and 4 days by ip injection did not affect SHP, HNF1α, HNF4α and LRH-1 mRNA in ileum and liver (data not shown).

Regulation of CYP3A, CYP3A2 and CYP3A9 expression in rat ileum and liver – in vivo. The administration of $1,25(OH)_2D_3$ to Wistar rats at 1200 pmol/kg/day for 12 h and 4 days by ip induced CYP3A1 expression in rat jejunum, ileum but not in colon (Figs. 6A, B, C and D)

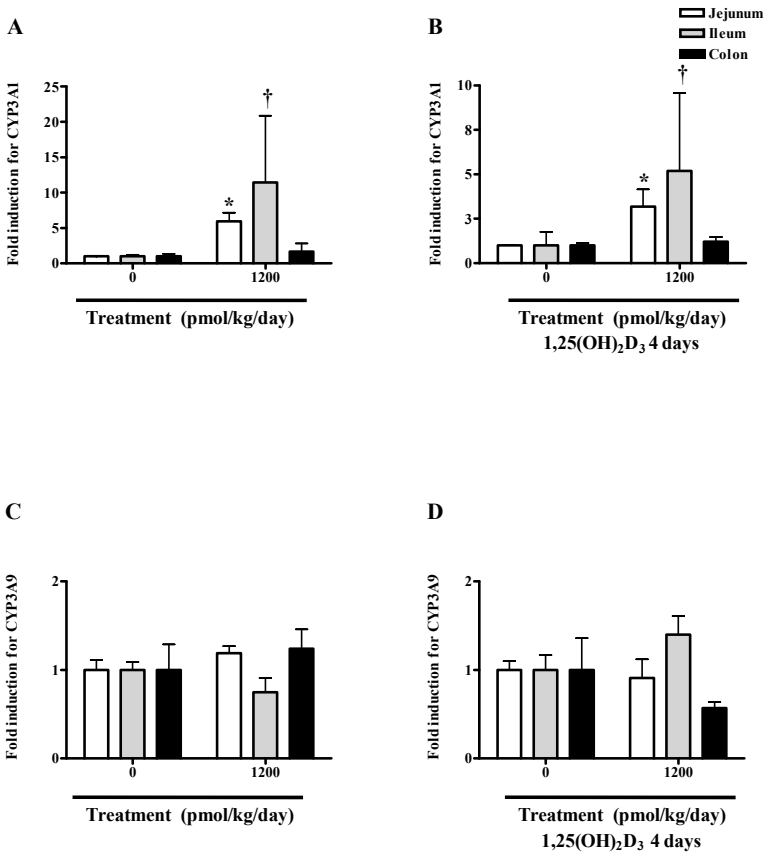

*Figure 6. Wistar rats were treated with with $1,25(OH)_2D_3$ (1200 pmol/kg/day) for 12 h and 4 days by intraperitoneal injection. After 12 h or 24 h of the last dose administered, rats were sacrificed and total RNA was isolated from jejunum, ileum and colon mRNA expression of CYP3A1 (A and B) and CYP3A9 (C and D) was evaluated by qRT-PCR. Results were expressed as fold-induction after normalizing with villin/GAPDH, and compared with the control rats treated with vehicle for the same duration of treatment.. Results showed mean ± S.E.M. of 3 to 6 rats. Significant differences towards the vehicle treated rats were indicated with * $P < 0.05$.*

Effect of 1,25(OH)$_2$D$_3$ treatment on portal and systamic bile acid levels. Total bile acid concentration was slightly elevated in the portal blood of Wistar rats treated with 1200 pmol/kg/day for 12 h and 4 days by ip injection compared to that of vehicle treated controls (35 µg/ml controls versus 40 µg/ml for 4 days). However, the difference failed o reach statistical significance.

Regulation of ASBT expression in human ileum and liver. In the human ileum, ASBT mRNA expression was found to be constant during 8 h of incubation, whereas levels were significantly decreased upon 24 h of incubation. The presence of the organic solvent vehicle failed to effect changes (Fig. 7A). Incubation of human ileum slices with 1,25(OH)$_2$D$_3$ significantly decreased the ASBT expression during 8 h of incubation (Fig. 7B), but no effect was seen after 24 h of incubation with 1,25(OH)$_2$D$_3$ (Fig. 7B). LCA and CDCA also decreased the ASBT expression transiently at 8 h of incubation and not after 24 h of incubation (Fig. 7B). DEX and BUD induced ASBT expression in human ileum slices at 8 h and 24 h (Fig. 7B). In human liver slices, the ASBT expression was significantly decreased with 24 h of incubation, and the results were not influenced by the organic solvent (Fig. 8A). 1,25(OH)$_2$D$_3$ did not affect ASBT expression (Fig. 8B). CDCA and LCA did not affect ASBT expression (Fig. 8B), whereas DEX induced ASBT mRNA expression 2.5-fold (Fig. 8B).

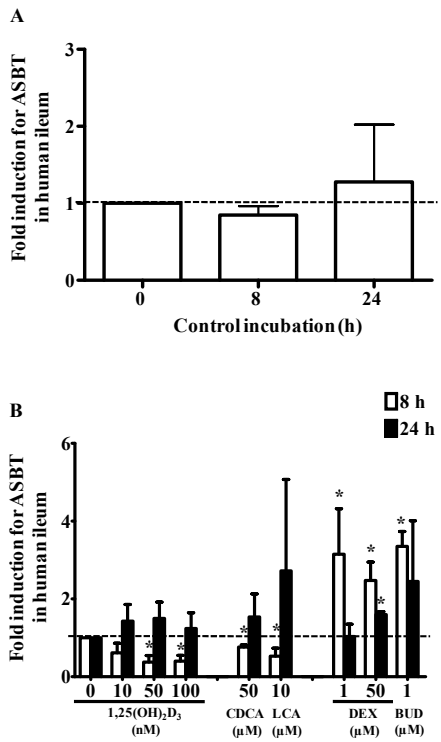

*Figure 7. Human ileum slices were exposed to control incubation (A)1,25(OH)$_2$D$_3$ (10-100 nM), CDCA (50 µM), LCA (10 µM), DEX (1-50 µM) and BUD (1 µM) (B) for 8 h and 24 h, after which total RNA was isolated and mRNA expression of ASBT was evaluated by qRT-PCR. The results were expressed as fold-induction after normalizing with villin and compared with the control incubated slices for the same length of time, which was set to unity. Results showed mean ± S.E.M. of 3-4 human ileum donors; in each experiment 3 ileum slices were incubated per condition. Significant differences towards the control incubations are indicated by * for P < 0.05.*

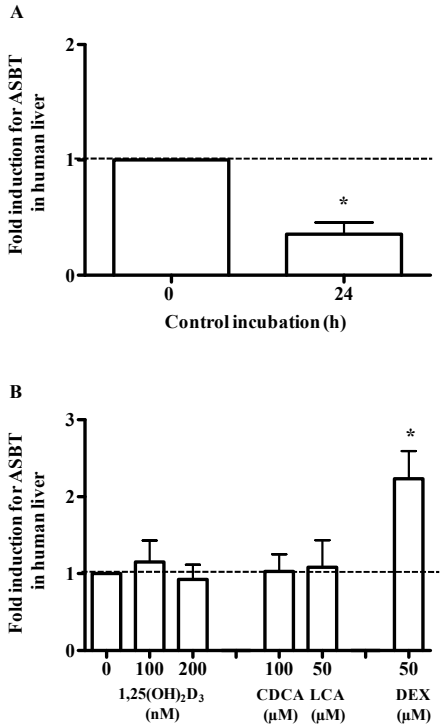

*Figure 8. Human liver slices were exposed to control incubation (A), 1,25(OH)$_2$D$_3$ (100-200 nM), CDCA (50 µM) and LCA (10 µM), DEX (50 µM) (B) for 24 h, after which total RNA was isolated and mRNA expression of ASBT was evaluated by qRT-PCR. Results were expressed as fold-induction after normalizing with GAPDH and compared with the control incubated slices for the same length of time, which was set to unity. Results showed mean ± S.E.M. of 3-5 human liver donors; in each experiment 3 liver slices were incubated per condition. Significant differences towards the control incubations are indicated by * for P < 0.05.*

Regulation of SHP, HNF1α, HNF4α and LRH-1 expression in human ileum and liver slices. The expression of SHP, HNF1α and HNF4α mRNA was found to decrease during control incubations of human ileum slices and not influenced by the solvents during 8 h and 24 h incubation (data not shown). Incubation of human ileum slices with 1,25(OH)$_2$D$_3$ did not affect SHP and HNF4α expression (Fig.9A and B), whereas HNF1α expression was significantly decreased (50 % expression at 100 nM 1,25(OH)$_2$D$_3$ vs. control incubated slices; $P < 0.05$) (Fig. 9A and B). CDCA and LCA induced SHP expression (4-5-fold induction; $P < 0.05$) (Fig 9A and B.) but did not affect HNF1α and HNF4α expression (Fig. 9A and B). DEX and BUD induced HNF1α and HNF4α and did not affect SHP expression in human ileum slices (Fig. 9A and B). In human liver slices, 1,25(OH)$_2$D$_3$ did not affect SHP and HNF1α expression but significantly decreased HNF4α expression (chapter 4). CDCA significantly induced SHP expression in human liver slices (chapter 4), whereas LCA induced SHP expression in three out of seven liver and was not consistent (chapter 3). CDCA and LCA decreased HNF1α and HNF4α expression (chapters 3 and 4).

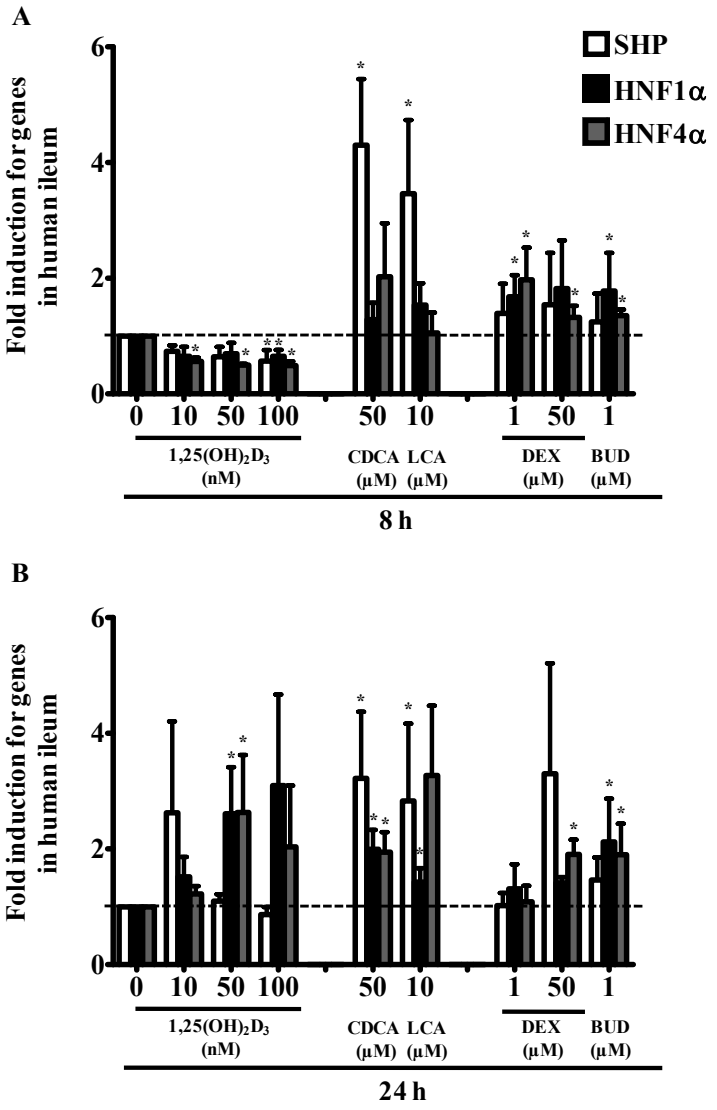

*Figure 9. Human ileum slices were exposed to 1,25(OH)$_2$D$_3$ (10-100 nM), CDCA (50 μM), LCA (10 μM), DEX (1-50 μM) and BUD (1 μM) (B)for 8 h (A) and 24 h (B), after which total RNA was isolated and mRNA expression of SHP, HNF1α and HNF4α was evaluated by qRT-PCR. Results were expressed as fold-induction after normalizing with villin and compared with the control incubated slices for the same length of time, which was set to unity. The results showed mean ± S.E.M. of 3-4 human ileum donors; in each experiment 3 ileum slices were incubated per condition. Significant differences towards the control incubations are indicated by * for P < 0.05.*

Discussion

The expression of the ASBT gene has been reported to be positively regulated by VDR, PPARα and GR ligands (11, 26, 38), and negatively regulated by the FXR ligands

(37). Most of the data on the regulation of ASBT in humans were obtained in cell lines, and data on human tissue is scarce. The available data show that there exists considerable interspecies difference, and extrapolation from animal studies to the human is hazardous. Moreover, reports on the ASBT regulation in the rat showed conflicting results. The rat ASBT was reported to be unresponsive to bile acids, FXR ligands, in the ileum due to a lack of LRH-1 binding site in the ASBT promoter (10), but was induced/repressed/no effects by bile acids in rat bile duct epithelial cells (cholangiocytes) (3, 17, 21, 41, 47). Therefore, in this communication, we studied and compared the direct effects of VDR, FXR and GR ligands on the regulation of ASBT in rat and human ileum and liver precision-cut tissue slices under identical experimental conditions. Further, we compared the *in vitro* effect of $1,25(OH)_2D_3$ on the regulation of ASBT in rat ileum and liver slices with those obtained *in vivo* by intraperitoneal treatment (ip) of Wistar rats with $1,25(OH)_2D_3$.

The VDR ligand, $1,25(OH)_2D_3$, significantly decreased the expression of ASBT both in rat and human ileum slices (Figs.1B and 7B). The $1,25(OH)_2D_3$ mediated repression of ASBT observed in human ileum slices was transient and was not found in slices incubated for 24 h (Fig. 7B). This might be attributed to the simultaneous induction of CYP3A4 in human ileum slices and Caco-2 cells by $1,25(OH)_2D_3$ (27, 42) and CYP24A1 (5), which inactivates $1,25(OH)_2D_3$ (54). The decrease in $1,25(OH)_2D_3$ mediated ASBT expression was unexpected as Chen et al. (11) characterized a VDRE in the rat ASBT promoter and reported $1,25(OH)_2D_3$-liganded VDR induction of ASBT in Sprague-Dawley rats. Since, the ileum tissue for *ex vivo* experiments was used from the Wistar rats, we studied the ASBT expression in the ileum of Wistar rats treated with $1,25(OH)_2D_3$ for 12 h and 4-days, and found no effect on the ASBT expression in the ileum of these rats, while a 2- fold induction was seen in the kidneys (Figs. 5A and B), which is similar to the unpublished observations of Chow et al. In addition these rats showed a induction of CYP3A1 in jejunum and ileum (Fig. 6), observations consistent with our earlier report on the tissue slices (27). Recently, Chow et. al. (12) found increased ASBT protein but without increase in ASBT mRNA in Sprague-Dawley rats treated with higher doses of $1,25(OH)_2D_3$ (2560 pmol/kg/day) for 4 days. We could not study the effect of $1,25(OH)_2D_3$ at a dose beyond 1220 pmol/kg/day, as reduced growth of the animals and significant increase in the weight of kidneys (data not shown) were detected at this dose, probably due to toxicity. The apparent difference in the $1,25(OH)_2D_3$ mediated ASBT expression *in vitro* and *in vivo* in the Wistar rat might be attributed to the differential exposure of the rat intestine to $1,25(OH)_2D_3$, but concentrations in the ileum could not be measured *in vivo*. The higher induction of CYP3A1 in jejunum versus ileum suggests a lower exposure of the ileum to $1,25(OH)_2D_3$ than the jejunum, as reported by Brown et al (7), because *in vitro* a higher induction of CYP3A1 in ileum slices was found compared to the jejunum slices, when they were exposed to $1,25(OH)_2D_3$ under identical experimental conditions (27).

In order to investigate the possible mechanism of this unexpected down regulation of ASBT by $1,25(OH)_2D_3$, we investigated the effect of $1,25(OH)_2D_3$ on the NR/transcription factors involved in the regulation of ASBT in rat and human ileum slices, and the ileum of Wistar rats treated with $1,25(OH)_2D_3$. In rat and human ileum slices $1,25(OH)_2D_3$ decreased the HNF1α expression without affecting SHP and HNF4α expression (Figs. 4 and 9), and the LRH-1 expression was not affected in rat ileum slices (Fig. 4). Thus, as HNF1α is essential for the basal expression of ASBT (43), the $1,25(OH)_2D_3$ mediated decrease in ASBT expression in rat and human ileum might be due to a decrease in HNF1α expression. The SHP, HNF1α, HNF4α and LRH-1 expression was not affected in the ileum of Wistar rats treated with $1,25(OH)_2D_3$ (data not shown). Recently, Chow et al (12) showed induction of SHP in the ileum of Sprague-Dawley rats treated with $1,25(OH)_2D_3$ at a dose of 2560 pmol/kg/day for 4 days and suggested to be mediated by the activation of FXR by the increased absorption of bile acids as a result of the increased ASBT protein. As we did not find significant elevated bile acids in the portal blood of Wistar rats treated with $1,25(OH)_2D_3$, an FXR activation was not expected. Hence, the results may indeed be dose-dependent or strain-dependent in the rat.

Subsequently, rat and human ileum slices were incubated with another VDR ligand, LCA (34). Similar to $1,25(OH)_2D_3$, LCA significantly decreased ASBT expression as well as HNF1α in rat and human ileum slices (Figs. 2A, 7B and 4). These results further confirm the involvement of VDR in the repression of ASBT in the rat ileum slices independent of FXR pathway. As LCA also shows affinity towards FXR, and induces SHP, we investigated the direct effects of the FXR ligand, CDCA on the ASBT expression in rat and human ileum slices. As expected, CDCA did not affect the ASBT expression in rat ileum slices (10), but significantly decreased ASBT expression in human ileum slices (37) (Figs. 2A and 7B). Both in rat and human ileum slices, CDCA induced the SHP expression (Figs. 4 and 9) showing an intact FXR pathway (18). The apparent differences in the regulation of ASBT in rat and human ileum slices, in spite of SHP induction, might be due to the reported absence of an LRH-1 binding site in the rat ASBT promoter, whereas it is present in the human ASBT, which is responsible for a FXR-SHP-LRH-1 mediated repression of ASBT (10, 18). In line with these findings, also the FXR synthetic ligand, GW4064 did not affect ASBT expression but induced SHP expression in rat ileum slices (Figs. 2A and 4), confirming the absence of a role of FXR in the regulation of rat ASBT. Further, CDCA induced HNF1α and HNF4α expression in rat and human ileum slices (Fig. 4 and 9). However, LRH-1 expression was not affected by CDCA in rat ileum slices (Fig. 4). To better mimic the *in vivo* situation where bile acids and $1,25(OH)_2D_3$ are present simultaneously, rat ileum slices were incubated with $1,25(OH)_2D_3$ in the presence of CDCA. The $1,25(OH)_2D_3$ mediated decrease in ASBT expression was not changed in the presence of CDCA (Fig. 2B), while SHP was induced (28).

Subsequently, we characterized the role of GR ligands, which are reported to induce ASBT expression in the rat and human ileum upon glucocorticoid treatment (25,

38). Furthermore, Jung et al. (25) reported a GRE in the rat ASBT promoter. As expected, DEX significantly induced the ASBT expression in rat and human ileum slices (Figs. 2A, 7A and 7B). In addition, DEX induced the HNF1α expression in rat and human ileum slices (Fig. 4, 9A and 9B). Hence, the GR ligand seems to induce ASBT expression by directly interacting with the GRE in the ASBT promoter through ligand activated GR, and also by inducing the HNF1α expression, which is reported to be essential for the basal expression of ASBT (43). In addition, DEX also induced SHP and LRH-1 expression in rat but not in human ileum slices (Figs. 4, 9A and 9B), which is not reported earlier. The presence of a GRE and the absence of a LRH-1 binding site in the rat ASBT promoter, together with the induction of HNF1α by GR ligands favour the induction of ASBT, in spite of simultaneous increase in SHP expression. Although, the LRH-1 expression was reported to be absent in the rat ileum (10), our studies clearly show that the LRH-1 mRNA is expressed along the length of the rat intestine and is further induced by the GR ligands in ileum (Fig. 4). The HNF4α expression was not affected by DEX in rat and human ileum slices. In human ileum slices, BUD, a synthetic GR ligand, similar to DEX also induced ASBT and HNF1α expression (Figs. 7B, 9A and 9B), confirming the involvement of the GR in the ASBT regulation. Recently, we reported the induction of the bile acid basolateral transporters, OSTα-OSTβ, by DEX and BUD in human ileum slices (28). Thus, simultaneous induction of ASBT and OSTα-OSTβ by glucocorticoids preserves the bile acid pool in crohns patients.

Subsequent to our studies in rat and human ileum on ASBT regulation, we also studied the regulation of ASBT in human and rat liver, where it is expressed on the apical surface of BEC and involved in the reabsorption of the conjugated bile acids from the bile ducts (53), although the physiological importance of this pathway remains obscure. $1,25(OH)_2D_3$ decreased the ASBT expression in rat liver slices, similar to rat ileum slices (Fig. 3). However, treatment of Wistar rats *in vivo* with $1,25(OH)_2D_3$ did not affect the ASBT expression in liver (Fig. 5), as also found by Chow et al. (12). It cannot be excluded that the BEC are exposed to only a very low fraction of $1,25(OH)_2D_3$ dose (7). In contrast to these findings in rat tissue slices, $1,25(OH)_2D_3$ did not affect the ASBT expression in the human liver (Fig. 8B), as opposed to the decrease in the human ileum (Fig. 7B). $1,25(OH)_2D_3$ also did not affect SHP, HNF1α and HNF4α expression in rat liver slices (chapter 4). However, incubation of rat liver slices with CDCA and LCA significantly induced ASBT expression, but GW4064 did not affect its expression (Fig. 3A and B) suggesting the absence of a role for FXR in the induction of ASBT in the rat liver. CDCA and LCA did not affect the ASBT expression in human livers (Fig. 8B), which is not reported before and suggests a species difference. Previously, we showed that CDCA and LCA induced SHP expression as expected and significantly decreased HNF1α, HNF4α and LRH-1 expression in rat liver slices (Chapter 3 and 4). In addition we showed that in human liver slices, CDCA but not LCA induced the SHP expression with subsequent repression of HNF1α, and HNF4α (Chapter 3 and 4). These results suggest that the ASBT expression in rat but not in human liver is positively regulated by bile acids in proportion to

their concentration. These results are in contrast to that of Kip et al. (29), who found a decrease in ASBT expression in the livers of taurocholate fed rats, but similar to the reports of Alpini et al. (1), who suggested that the bile acid- mediated induction of ASBT in rat livers might be mediated via protein kinase C (PKC), since bile acid are reported to activate PKC by phosphorylation (31). However we did not find an induction of ASBT in rat liver slices incubated with the PKCα agonist, phorbol-12-myristate-13-acetate (PMA) (data not shown). Furthermore, the GR ligand, DEX significantly induced ASBT expression in rat and human liver slices similar to that of ileum slices. Previously we found that DEX decreased the HNF1α expression but induced the SHP and LRH-1 expression without affecting HNF4α in rat liver slices (Chapter 2 and 3), and that DEX induced the HNF4α expression without affecting the SHP and HNF1α expression in human liver slices (Chapter 2 and 3). The changes in the SHP, HNF1α, HNF4α and LRH-1 expression in response to the bile acids and the GR ligands in rat and human liver seem to be restricted to the hepatocytes, whereas ASBT is expressed in the chaolangiocytes, which may explain the absence of an influence on the regulation of ASBT.

In summary, we have characterized the direct effects of VDR, FXR and GR ligands in the regulation of ASBT in rat and human ileum and liver and compared them with the *in vivo* effects of $1,25(OH)_2D_3$ in Wistar rats. We found a clear species and organ specific difference in ASBT regulation. In the rat and human ileum slices, the VDR ligands $1,25(OH)_2D_3$ and LCA decreased the ASBT expression possibly due to a repression of HNF1α. The FXR ligand, CDCA decreased the ASBT expression in human ileum slices. In rat, FXR-liganded ASBT repression via the FXR-SHP-LRH-1 pathway is not observed in the presence of CDCA and GW4064, which is in line with a lack of LRH-1 binding site in the rat ASBT promoter. Furthermore, in contrast to rat and human ileum, CDCA induced ASBT expression in rat but not in human liver, despite an intact FXR pathway. As expected, the GR ligands induced the ASBT expression in rat and human ileum and liver, indicating an intact GR pathway in these tissue slices. In conclusion, our results show a clear species and organ specific difference in the regulation of ASBT.

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