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

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Regulation of Metabolizing Enzymes and Transporters for Drugs and Bile Salts in Human and Rat Intestine and Liver

- A study with precision-cut slices

Ansar Ali Khan

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Regulation of Metabolizing Enzymes and Transporters for Drugs and Bile Salts in Human and Rat Intestine and Liver

A study with precision-cut slices

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Paranimfen: Kirankumar Katta
Marlies Schippers

Dedicated to my parents, wife and kids

Table of contents

Chapter 1
Introduction: Scope of the Thesis

Ansar A. Khan

Geny M. M. Groothuis

1 Introduction

Both the liver and the intestine play a vital role in determining the bioavailability of orally ingested therapeutic drugs. They also play a role in the elimination of potentially harmful exogenous and endogenous compounds from the blood. The role of the liver in first-pass metabolism and as one of the limiting factors in determining the oral bioavailability of drugs is well appreciated due to the high expression of a wide range of phase I and phase II drug metabolizing enzymes (DME) and drug transporters (DT) (27, 34, 94). During the past 20 years, the role of the intestine as an important factor in determining first pass metabolism of drugs is increasingly recognized as a result of the accumulation of data on intestinal metabolism and on the expression and identification of DME's and DT's (4, 31, 61, 69, 117, 136). The expression of the cytochrome P450 (CYP) family has been extensively studied in both human and rat intestine. In the human intestine CYP3A is the most abundant of the CYP isozymes (59-94% of which 33-87% is CYP3A4), followed by CYP2C9 (4-38%), CYP2C19 (0.5-7%), CYP2J2 (0.2-4%), CYP2D6 (0.2-4%) (142). Moreover CYP1A1 and CYP2S1 are found to be expressed in the human intestine. The expression of CYP isozymes in the intestine is different from that in the liver in which CYP3A4 and CYP3A5 are the most abundant CYPs, but representing only 40% of the total CYP P450, followed by CYP2C9/19 (25%), CYP1A2 (18%), CYP2E1 (9%), CYP2A6 (6%), CYP2D6 (2%) and CYP2B6 (<1%) (109). In the rat intestine, CYP3A is the most abundant CYP P450 isozyme and at least five different isoforms were found to be expressed: CYP3A1 (45), CYP3A2 (45, 133), although at a low level, CYP3A9 (highly expressed in the intestine compared to the liver) (132), CYP3A18 (132) and CYP3A62 (89). CYP3A9 is expressed higher in female rats than in male whereas CYP3A18 is higher expressed in male. As CYP3A isozymes are reported to be involved in the biotransformation of 50-60% of the therapeutic drugs (44) and as the intestine is richly endowed with CYP3A isozymes, it may be evident that intestine plays a vital role in drug metabolism. The clinical significance of the intestine in first pass metabolism is documented by its role in determining the oral bioavailability of cyclosporine, midazolam and verapamil as demonstrated in *in vivo* studies (40, 68, 145). Apart from the CYP isozymes, the expression of flavin monooxygenases (FMO), 17β-hydroxysteroid dehydrogenase (HSD) and many phase II DME's, particularly uridinediphosphate glucoronyltransferases (UGT's), sulphotransferases (SULT), glutathione S-transferases (GST) and methyltransferases (MT) have also been reported in the intestine (12, 52, 106-108, 116).

In the intestine, drug absorption and metabolism take place in the enterocytes, where the DMEs and DTs are located. The enterocytes are epithelial cells lining the luminal surface of the intestine. They are polarized cells with a clear marginalization of the basolateral and apical membranes with a distinct set of transport proteins. The transport proteins function as uptake and efflux carriers or pumps and play a role in the vectorial transport of nutrients and drugs across the epithelial cells either from the lumen into the

blood or from the blood into the lumen (61). The major transporters have been classified as members of the solute carrier family (SLC) and the ATP-binding cassette (ABC) protein family, according to the classification by the Human Genome Organization (HUGO). The expression of phase I, and phase II DME's and DT's varies along the length of the intestine as well as along the crypt to villous axis, with the highest expression in the villous tip in the particular segment of the intestine. The distribution of various phase I and phase II DME's and DT's along the length of the intestine has been summarized by Pang (112) and the interspecies differences in the expression of these proteins in rat and human intestine is recently reviewed by van de Kerkhof et al.(142). Some of the DME's and the DT's are present at the highest concentration in the proximal part of the small intestine with decreasing gradient along the intestinal tract, whereas others show an increasing gradient or an even distribution. In man, CYP3A4 expression is declining along the small intestine, CYP2S1 protein is equally expressed along the intestinal tract (125), whereas the expression of CYP2J2 increases along the small intestine (150). In the proximal part of the human small intestine CYP3A4 is the predominant enzyme, whereas in the large intestine CYP3A5 is more abundant (115). Similar to man, also in the rat intestine the metabolic activity of the phase I DME's is usually higher in the duodenum and the jejunum than in the ileum and the colon (133, 141). 7-ethoxy coumarin O-deethylation, mediated predominantly by CYP1A (141), and androstenedione formation from testosterone mediated mainly by HSD decreases in distal direction (35). In contrast, CYP3A9 and CYP3A18 mRNA expression first increases and then remains constant along the small intestine (132). The distribution pattern of NADPH-dependent cytochrome P450-reductase activity, which is required for the CYP450 reactions (87), closely parallels that of CYP3A (110). In contrast, mucosal microsomal cytochrome b5 protein content and cytochrome b5 reductase activity (enhancers of the monooxgenase reaction) tend to increase slightly in distal direction (110). Similar to the CYP P450 isozymes, the Phase II conjugation enzymes are also not equally distributed along the rat intestinal tract. Sulphation rates decrease in distal direction in the small intestine (12, 106), but the highest activity has been found in the colon (141). In the small intestine, the UGT2B1, UGT2B3 and UGT2B6 expression decreases in distal direction (116) (127), whereas UGT1A1 and UGT1A6 activities are homogeneously distributed along the small intestine (133). However, in the colon, UGT1A1, UGT1A3, UGT1A6 and UGT1A8 expressions are higher than in the small intestine (127), which is in line with activity data showing that 7-hydroxycoumarine glucuronidation (mainly UGT1A6) is higher in the colon than in the small intestine (141). The GST activity shows a decreasing gradient in distal direction along the rat small intestine (102) as well as from proximal to distal colon in man (52).

Drug uptake and efflux transporters, such as PEPT1 (139), multidrug resistance associated protein (MRP)2 and multidrug resistance protein (Pgp; MDR) (38) also exhibit a gradient of expression along the length of the intestine (112). The most important transporters detected in human and rat intestine have recently been reviewed by van de Kerkhof et al. (142). In the human intestine, the expression of some transporters increases

from proximal to distal such as MDR1 (95, 152, 154), MRP1 (154), organic cation transporter (OCTN)2 (92), whereas the expression of breast cancer resistance protein (BCRP; ABCG2) (46), MRP2 (33, 154), concentrative nucleoside transporter (CNT)1-2 (92), serotonin transporter (SERT) (92), PEPT1 (33, 92) and OCTN2 (33, 92) decreases or remains constant along the length of the intestine as was also reported for organic anion-transporting polypeptide (OATP)2B1 (33, 92) and OCTN1 (92). Furthermore, MRP3 is expressed highest in the colon (33, 154) and the expression of ASBT and the organic solute transporter (OST)α-OSTβ is higher in the ileum than in both the duodenum and the colon (55, 63, 92). In rat intestinal tissue, several transporters have been detected. Similar to human intestine, rat Mrp2 expression decreases in distal direction along the tract (16, 122). In contrast, Bcrp, Mdr1a, Mdr1b and Mrp3 expression increases along the length of the intestinal tract (6, 11, 122, 132, 135). Like in human also in the rat ASBT and OSTα-OSTβ are highest in the ileum compared to the jejunum and the colon (6, 63).

The expression of phase I and phase II DME's and DT's in the human intestine exhibits significant inter-individual variation in the population, which can be attributed to natural phenomena such as genetic polymorphism, gender-specific variation, and age etc (75, 91, 130). Furthermore, life style, diet and disease are also sources of variation. The activity of DME's and DT's is subjected to alterations in response to induction and inhibition stimuli, which have significant impact on the bioavailability of oral drugs, resulting in either increase or decrease in efficacy or toxicity. Drug–drug interactions (DDIs) are often due to the concomitant effect of a co-administered drug on the absorption or elimination pathways. These DDIs can be the result of inhibition of the uptake or efflux transporters or metabolizing enzyme or of induction or inhibition of their expression (48, 79, 100, 146). Classical examples are the inhibitory effect of furanocoumarins, components of grapefruit juice and gemfibrozil on the metabolism of CYP3A4 substrates such as felodipine and rosiglitazone leading to an increased bioavailability of these drugs in hypertensive and diabetic patients, respectively (5, 101).

There are many mechanisms involved in the regulation of the expression of DME's and DT's, and constitutive, induced and repressed expression of genes is largely controlled at the level of transcription by the involvement of nuclear receptors, such as pregnane X receptor (PXR), constitutive androstane receptor (CAR), glucocorticoid receptor (GR), vitamin D receptor (VDR) and aryl hydrocarbon receptor (Ahr) and Nrf2 (66, 67, 88, 96, 114, 138), which are expressed in various gradients along the length of the intestine (64, 151). The intestinal DME's and DT's are suggested to be much more responsive to inducing stimuli than those in the liver (79). This may be due to the fact that in vivo, the intestinal DME's serve as a protective barrier and abort the effect of various orally ingested inducers on hepatic DME's (144). Moreover after oral dosing, the intestines are exposed to much higher concentrations compared to the liver. Thus, apart from its physiological role of absorption of nutrients, the intestinal mucosal tissue with the expression of various DME's and influx and efflux DT's, also plays a vital role in the

detoxification of exogenous and endogenous compounds and acts as a selective and responsive barrier for the exposure to orally taken molecules (31).

2 Bile acids – synthesis, transport and the enterohepatic cycle

Bile acids are synthesized from cholesterol. This conversion of cholesterol to bile acids (BA) is mediated by a cascade of 12 enzymatic reactions (124) involving two pathways, the classic or neutral pathway and the acidic or alternative pathway. The neutral pathway is the major pathway of bile acid synthesis and it is exclusively hepatic, initiated by the first and the rate limiting step, cholesterol 7α-hydroxylation, catalyzed by the microsomal enzyme CYP7A1 (18), which results in the formation of two primary bile acids, chenodeoxycholic acid (CDCA) and cholic acid (CA). The acidic pathway is initiated by CYP27A1-catalyzed 27-hydroxylation of cholesterol. This enzyme is expressed in many tissues and plays an important role in the reverse cholesterol transport from peripheral tissues to the liver (17). In the liver, primary bile acids are amidated with glycine or taurine and are subsequently transported into the bile canaliculi by the bile salt export pump (BSEP; ABCB11) and secreted into the intestine via bile. About 95 % of the bile acids are actively absorbed in the terminal ileum by the coordinate action of sodium dependent apical bile acid transporter (ASBT; SLC10A2) and the basolateral heterodimeric OSTα-OSTβ (7, 23, 24, 37, 120, 128) into the portal circulation. Bile acids are also substrates for OATP2B1 but its role in uptake of bile acids in the intestine seems to be minimal. These bile acids are then transported to the liver and taken up by the hepatocytes by the sodium dependent co transporting polypeptide (NTCP; SLC10A1) and by OATP's (71, 72). Altogether, bile acid synthesis in the liver and excretion into the bile canaliculi, secretion into the intestine via the bile ducts, reabsorption in the ileum and transport back to the liver via the portal circulation and finally uptake by the hepatocytes constitutes the enterohepatic cycle (13, 53, 60, 129), which maintains the bile acid pool *in vivo*.

3 Bile acids - endogenous toxicants and biotransformation pathways.

Bile acids per se are toxic, when presented to the cells in high concentrations, but under normal conditions the above mentioned transporters keep the intracellular concentrations at non-toxic levels. During passage along the intestine, a part of the primary bile acid pool is subjected to 7-α dehydroxylation by bacterial flora in the terminal part of the small intestine, resulting in the formation of monohydroxy, hydrophobic toxic secondary bile acids, deoxycholic acid (DCA) from CA and lithocholic acid (LCA) from CDCA (22, 51). These bile acids, with the exception of LCA are efficiently reabsorbed in the ileum by ASBT as a part of the enterohepatic cycle and transported back to the hepatocytes. Among the secondary bile acids, LCA is the most toxic and hydrophobic, and it is passively absorbed by the intestinal mucosal cells in the terminal ileum and the colon (70, 126, 134). LCA is reported to be a cholestatic agent in the liver and carcinogenic in the intestine in animals and man (36, 59, 97), which is due to its potential to form DNA

adducts and to inhibit DNA polymerase-II, a DNA repair enzyme (47, 103). LCA is efficiently metabolized by CYP enzymes in humans (CYP3A4) and rats (CYP3A1, CYP3A2, CYP3A9, CYP2C6, CYP2C11 and CYP2D1) to 6α- and 6β-hydroxy metabolites, respectively (3, 29, 80, 155). The major products of LCA metabolism in human and rat liver microsomes are hyodeoxycholic acid (HDCA), murideoxycholic acid (MDCA), 3-keto-5β-cholanic acid (3KCA) (10).and CDCA (147). Although intestinal metabolism is considered important for the detoxification, LCA metabolism was mainly studied in liver microsomes and no data is available on intestinal metabolism.

Bile acids can bind and activate the nuclear receptors involved in the regulation of the expression of DMEs and DTs (41, 43, 82, 137). This suggests that bile acids interact with the absorption, metabolism and disposition of therapeutic drugs which may be different under normal and pathophysiological conditions such as liver diseases like cholestasis or intestinal diseases like crohns disease.

4 The role of nuclear receptors in bile acid synthesis, transport and detoxification

Gene expression is generally under control of nuclear receptors (NR). There are currently 48 nuclear receptors identified in human, 49 in mice and 47 in rats that are categorized into six subfamilies: NR1-NR6 (121). NR1 family includes the thyroid hormone receptor like, the NR2 family includes the retinoid receptor like, the NR3 family the esterogen receptor like, the NR4 family includes the nerve growth factor IB like, the NR5 family the steroidogenic factor like, and the NR6 family contains the germ cell nuclear factor like NRs. The rest is classified as NR0 containing miscellaneous NRs (39). The nuclear receptors in the NR1 and NR2 families are important in bile acid homeostasis and regulate the transcription of transporters and enzymes as well as that of other nuclear receptors, with the farnesoid X receptor (FXR; NR1H4) being the most important in governing bile acid homeostasis (83). Apart from these NRs, expression of these genes is influenced by nuclear factors that serve as co-activators or repressors.

4.1 Regulation of bile acid synthesis

As already described above, the rate of bile acid synthesis is regulated by the expression of the microsomal enzyme CYP7A1, catalyzing the first and rate limiting step in the conversion of cholesterol to bile acids in the hepatocytes (18). The CYP7A1 expression is induced by oxysterols, derived as intermediates in the cholesterol and bile acid synthesis pathways (77) and repressed by bile acids such as CDCA and CA, the end products of bile acid synthesis in the hepatocytes (42). The CYP7A1 promoter contains two bile acid response elements (BARE's), BARE-I and BARE-II, that both contain AGGTCA-repeat sequences which are arranged as direct repeats (DR)1, DR3, DR4 and DR5, inverted repeats (IR)1 and everted repeats (ER)6 (17). Many transcription factors have been identified, which bind to BARE's either as a monomer, like liver receptor homologue 1

(LRH-1; NR5A2), as a homodimer, like hepatocyte nuclear factor α (HNF4α; NR2A1) or as a heterodimer with retinoic acid X receptor α (RXRα; NR2B1), such as liver X receptor α (LXRα; NR1H3), FXR, peroxisome proliferator activated receptor (PPAR's; NR1C), retinoic acid receptor (RAR; NR1B) and PXR. HNF4α and LRH-1 bind to their respective elements in the promoters in a ligand-independent manner and are essential for the expression of their target genes, CYP7A1 and NTCP respectively. FXR and PXR are activated by bile acids such as CDCA, CA, DCA and LCA (113), LXRα is activated by oxysterols (77) and RAR is activated by 9-cis retinoic acid (1). Ligand-activated FXR heterodimerizes with the retinoic acid X receptor α (RXRα, NR2B1) and indirectly inhibits CYP7A1 and NTCP expression in the liver and ASBT expression in the ileum (28, 42, 98), by inducing the synthesis of the short heterodimer protein (SHP; NR0B2). SHP eventually inhibits the trans-acting factor, LRH-1, that is essential for the basal expression of these genes (14, 42, 76). In contrast, BSEP is positively regulated by ligand-bound FXR by direct binding to the IR1 motif in the BSEP promoter (2). In addition to this SHP-dependent pathway, CYP7A1 is also negatively regulated by a SHP-independent pathway mediated through activated c-Jun kinase. Transforming growth factor β (TGFβ) and other cytokines (25, 78), as well as FGF15, synthesized in the intestine under the control of CDCA-activated FXR (54, 57, 65), activate c-Jun kinase through protein kinase C thereby inactivating HNF4α by phosphorylation, which is essential for the basal expression of CYP7A1 (19, 123). Further, CYP7A1 mRNA and protein is subjected to posttranscriptional and posttranslational regulation (99). CYP7A1 expression is also positively regulated by oxysterols, and retinoic acid, that are ligands for LXRα, RXRα and RAR, which upon ligand binding heterodimerize with RXRα and bind to the DR4 or 5 motifs in the CYP7A1 promoter and induce its expression (20, 77). The role of LXRα in the regulation of CYP7A1 in man seems to be redundant because the DR4 motif is absent in its promoter (77).

4.2 Regulation of bile acid detoxification proteins

As described earlier, primary and secondary bile acids are potentially toxic when presented to the intestinal and liver cells in high concentration (97). They are detoxified by various phase I and phase II enzymes and further excreted by several ABC transporters. The expression of these proteins is altered in response to an increased or decreased bile acid concentration resulting from physiological (high fat diet leading to the increased bile flow) and pathophysiological (cholestasis) conditions. Recently, the vitamin D receptor (VDR; NR1I1), which belongs to the steroid/thyroid hormone nuclear receptor super family, NR1I1, and exhibits significant homology with PXR and CAR (84) is reported to modulate xenobiotic metabolism and transport. 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$ or calcitriol) (30), the active form of vitamin D is a natural ligand of VDR. In addition, VDR was shown to bind bile acids such as LCA with high affinity and induced CYP3A enzymes in mice and human liver and intestine (82) serving to detoxify toxic bile acids (131). Therefore VDR is regarded as a bile acid sensor in the intestine (82). The role of VDR in

the regulation of DMEs and DTs in the human and rat liver and intestine is not completely understood but ligand activated VDR is reported to induce human CYP3A4 in human intestinal cell lines as Caco-2 and LS180 (137), CYP2B6 and CYP2C9 (121), and rat CYP3A9 (153) and CYP3A1/CYP3A23 (148). In addition, $1,25(OH)_2D_3$ was found to induce the expression of murine multidrug resistance-associated protein 3 (MRP3; ABCC3) (90) and CYP24A1 in the kidney (49), human hydroxysteroid sulfotransferase, SULT2A1 (32), and the ASBT in the rat intestine (15).

5 Models to study regulation of bile acid synthesis and detoxification proteins

The effects of ligands of the various NR on bile acid synthesis and disposition proteins were studied in vivo either by interfering with bile acid uptake using resins such as cholysteramine, by duodenal infusion of bile acids and also by inhibiting the bile acid uptake using ASBT inhibitors (9, 50). Bile duct ligated and LCA induced cholestasis animal models (8), and human cholestatic livers were also used as valuable tools to investigate the effects of bile acids (156, 157). Recently, knockout out mice such as cyp7a1$^{-/-}$, asbt$^{-/-}$, shp$^{-/-}$, ntcp$^{-/-}$, mrp3$^{-/-}$, mrp4$^{-/-}$, Ostα$^{-/-}$, FXR$^{-/-}$, PXR$^{-/-}$, LXRα$^{-/-}$ (23, 62, 85, 93, 119, 120, 131, 149) were used to elucidate the role of these genes in bile acid synthesis and disposition and their role in physiology and pathophysiology. However, various confounding factors are associated with such models *in vivo* such as vitamin deficiency associated with cyp7a1$^{-/-}$ knock out animals which do not allow to discern the direct effects of ligands and the role of nuclear receptors from indirect effects.

In vitro studies like reporter gene assays are applied in characterizing the nuclear receptor response elements in the promoters. In addition cell cultures studies using primary cultures of rat and human hepatocytes and enterocytes, and immortalized human cell lines such as HepG2, LS180 and Caco-2 are widely applied to study the effect of various ligands on the bile acid synthesis and disposition proteins (21, 56, 58, 74, 104, 111). Rat and human hepatocyte sandwich cultures were also successfully used in characterizing the role of NR in bile acid synthesis enzymes and disposition proteins (118). However, primary cells in culture are not stable with respect to the expression of enzymes, transporters and nuclear factors and moreover immortalized cell lines do not exhibit normal expression of DME's, DT's and NR's (73, 142). In addition immortalized intestinal cell lines such as Caco-2, LS180 and IEC-6 do not reflect the segmental expression of DME's and DT's and the gradients of their activities along the length of the rat intestine (81, 141). The induction and repression of DME's and DT's in the intact intestinal and liver tissue in response to ligands of the NRs is dependent not only on the presence of NR response elements in the target genes but also on the expression levels of the NRs, co-activators and repressors and on the exposure of the particular cell to the ligand. This exposure is the result of uptake, metabolism and excretion of the ligand and its metabolites and may differ between the various regions of the intestine and the liver as a result of differences in the expression of uptake and efflux transporters and metabolizing enzymes. Furthermore, *in vivo*, different

regions of the intestine and liver are exposed to different concentrations of the ligands. To appreciate the potential differences of the effect of various ligands for various nuclear receptor pathways in different organs within the same species and between species, the effect of ligands needs to be studied under identical conditions in *in vitro* models using intact tissue.

Recently, the intestinal and liver precision-cut slice model was validated as an adequate *in vitro* model to study drug metabolism and induction of drug metabolizing enzymes and transporters using ligands of various nuclear receptors such as FXR, PXR, CAR; NR1I3 and Ahr. The effects of these ligands were assessed by measuring the activity of the induced CYP P450 enzymes using probe substrates as well as at the level of mRNA by quantitative real time PCR (qRT-PCR) both in rats, mice and human (26, 86, 105, 140, 143). The most important feature of the slices is the adequate representation of all the cell types in its natural and physiological environment. They can be prepared and cultured for 24 h from all regions of the intestine and from the liver, allowing the comparison of the effects of ligands under identical conditions in man and animals.

6 Aim of the thesis

The research described in this thesis is aimed to study **induction and repression of metabolizing enzymes and transporters involved in drug and bile acid detoxification, transport and synthesis in human and rat liver and intestine. Precision-cut tissue slices prepared from human and rat intestine and liver were used as a *in vitro* model.** The main focus was on the VDR mediated effects by $1,25(OH)_2D_3$ and LCA. Further, to obtain more insight, the effects of the VDR ligands were compared with those of specific ligands for other nuclear receptors, CDCA and GW4064 for FXR, pregnenolone-16α carbonitrile (PCN) for PXR, budesonide (BUD) for GR and dexamethasone (DEX) for GR and PXR. Apparent interspecies and organ-specific differences in the regulation of DMEs and DTs were observed and discussed.

In **chapter 2**, we investigated the gradient of expression of several NRs in the rat intestine and the effects of the VDR ligand, $1,25(OH)_2D_3$ on the expression of various CYP3A isoforms in the rat small intestine (jejunum and ileum), colon and liver, and of CYP3A4 in human ileum and liver slices, and compared it with that of specific PXR, FXR and GR ligands.

In **chapter 3**, we studied the effects of the toxic and cholestatic bile acid LCA, which is reported as a ligand for VDR, PXR and FXR, on the regulation of DME's and DT's in rat and human intestine and liver.

In **chapter 4,** we addressed the direct and indirect effects of $1,25(OH)_2D_3$ on bile acid synthesis and disposition proteins in rat and human liver at the level of mRNA by exposing the slices to the VDR and FXR ligands, $1,25(OH)_2D_3$ and CDCA, respectively.

In **chapter 5**, we studied the regulation of ASBT by natural and synthetic ligands for FXR, GR, VDR and PXR in rat and human ileum and liver tissue using precision-cut slices.

Subsequently, in **chapter 6** we investigated the effects of FXR, GR, VDR and PXR on the expression of OSTα-OSTβ, recently identified as the basolateral bile acid efflux transporter in the small intestine. This transporter is reported to be expressed along the length of the intestine in parallel to the bile acid uptake protein ASBT and in the cholangiocytes of the liver.

In **chapter 7,** we investigated the regulation of the VDR itself and more specifically the possible feedback / feed forward loops involved in this regulation as a result of cross talk between various nuclear receptors in rat and human intestine and liver. Moreover we studied the possible implications for the regulation of DME's and DT's.

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Chapter 2

Comparison of effects of VDR versus PXR, FXR and GR ligands on the regulation of CYP3A isozymes in rat and human intestine and liver

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Abstract

In this study, we compared the regulation of CYP3A isozymes by the vitamin D receptor (VDR) ligand, 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) against ligands of the pregnane X receptor (PXR), the glucocorticoid receptor (GR) and the farnesoid X receptor (FXR) in precision-cut tissue slices of the rat jejunum, ileum, colon and liver, and human ileum and liver. In the rat, $1,25(OH)_2D_3$ strongly induced CYP3A1 mRNA, quantified by qRT-PCR, along the entire length of the intestine, induced CYP3A2 only in ileum but had no effect on CYP3A9. In contrast, the PXR/GR ligand, dexamethasone (DEX), the PXR ligand, pregnenolone-16α carbonitrile (PCN), and the FXR ligand, chenodeoxycholic acid (CDCA), but not the GR ligand, budesonide (BUD), induced CYP3A1 only in the ileum, none of them influenced CYP3A2 expression, and PCN, DEX and BUD but not CDCA induced CYP3A9 in jejunum, ileum and colon. In rat liver, CYP3A1, CYP3A2 and CYP3A9 mRNA expression was unaffected by $1,25(OH)_2D_3$, whereas CDCA decreased the mRNA of all CYP3A isozymes; PCN induced CYP3A1 and CYP3A9, BUD induced CYP3A9, and DEX induced all three CYP3A isozymes. In human ileum and liver, $1,25(OH)_2D_3$ and DEX induced CYP3A4 expression, whereas CDCA induced CYP3A4 expression in liver only. In conclusion, the regulation of rat CYP3A isozymes by VDR, PXR, FXR and GR ligands differed for different segments of the rat and human intestine and liver, and the changes did not parallel expression levels of the nuclear receptors.

Keywords: cytochrome P450, induction, intestinal slices, liver slices, 1α,25-dihydroxyvitamin D_3

Introduction

The cytochrome P450 enzymes constitute a family of heme protein oxygenases that display considerable similarities in their molecular weights, immunohistochemical properties, and substrate specificities (9). The CYP3A isoforms play an important role in oxidation of endogenous steroids and toxic hydrophobic bile acids. In the rat, the CYP3A family consists of five isoforms: CYP3A1/CYP3A23, (11), CYP3A2 (10), CYP3A9 (43), CYP3A18 (35) and CYP3A62 (29). These enzymes are expressed predominantly in the liver and in the enterocytes of the intestine (21). The distribution of CYP3A isozymes in the rat appears to be sex-, tissue- and age-dependent. CYP3A2 and CYP3A18 are predominantly expressed in male rats (10, 30, 35), while CYP3A9 and CYP3A62 expression is higher in female rats. CYP3A1 and CYP3A2 are predominantly expressed in the rat liver, and CYP3A62, in female livers (29), whereas CYP3A9 is highly expressed in the intestine relative to the liver (27, 44). The human CYP3A family which is expressed in the liver is composed of at least four isozymes: CYP3A4, CYP3A5, CYP3A7 and CYP3A43 of which CYP3A4 is the predominant isozyme expressed in adult human liver (13). CYP3A4 and CYP3A5 isozymes are present along the human digestive tract, with CYP3A5 mainly present in the stomach and CYP3A4 along the intestine segments (21).

The expression of CYP3A isoforms in rats and humans was reported to be modulated by exogenous and endogenous ligands through the pregnane X receptor (PXR) (26), the glucocorticoid receptor (GR) (18), and the vitamin D receptor (VDR) (28, 37, 45). Recently, a FXR response element (FXRE) was found in the human CYP3A4 promoter, and induction by CDCA, a FXR ligand, was noted (8). The 5´ flanking promoter regions of the rat and human CYP3A are characterized by direct repeats spaced by three base pairs (DR3) and everted repeats spaced by six base pairs (ER6) (8, 14, 37). PXR, FXR and VDR directly bind to the respective response elements pursuant to the ligand binding and heterodimerization with retinoic acid X receptor α (RXRα) (8, 23, 36). In contrast, the GR effects on CYP3A isozymes in rat and humans have been attributed indirectly to the induction of HNF4α and PXR (16).

The effects of various ligands on rat and human CYP3A enzymes in the intestine and liver have been studied *in vitro* in both primary cultured hepatocytes and enterocytes, and immortalized human cell lines such as HepG2 and Caco-2 cells. Immortalized intestinal cell lines derived from the different regions of the rat intestine were utilized to study the regulation of drug metabolizing enzymes (46). However, these cell lines lack the normal expression of nuclear receptors (NRs), metabolic enzymes and transporters. For example, Caco-2 cells are PXR-deficient and exhibit reduced levels of drug metabolizing enzymes (24). Furthermore, cell lines are unable to reflect the segmental expression of CYP3A isozymes and the gradients of activities along the length of the rat intestine (25, 39). The induction / repression of CYP3A isoforms in the intact liver and intestinal tissue in response to ligands of the NRs have not been extensively investigated. Such a response is

dependent not only on the presence of NR response elements, but also on the expression levels of the NRs and exposure of the particular cell to the ligand. This exposure is the result of uptake, metabolism and excretion of the ligand and its metabolites and may differ between the various regions of the intestine and the liver as a result of differences in expression of uptake and excretion transporters and metabolizing enzymes. Different regions of the intestine and liver are exposed to different concentrations of the ligands *in vivo*. For an appreciation of the potential variation between the different organs and their sensitivity towards the NR ligands, studies should be conducted under identical conditions among these organs or tissues.

Therefore, in this study, we compared the effects of various NR ligands on the intestine and liver of the rat and human in precision-cut tissue slices. This model has been previously validated as a useful *ex vivo* model for induction studies (31, 40, 41) that enables us to investigate the effects of inducing ligands under identical incubation conditions for the liver and intestine. We tested the hypothesis that the regulation of rat and human CYP3A isozymes by VDR ligands differed from those by PXR, GR and FXR ligands. We compared the induction potential of PXR, FXR and GR ligands to that of VDR ligand, 1α,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) on changes in mRNAs of the various CYP3A isoforms in the small intestine (jejunum and ileum), colon and liver of the rat and the CYP3A4 in human ileum and liver slices, and investigated whether these responses correlated to the expression levels of the NRs.

Materials and methods

Chemicals and reagents. 1,25$(OH)_2D_3$ in ethanol was purchased from BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA. Chenodeoxycholic acid was purchased from Calbiochem, San Diego, CA, dexamethasone was from Genfarma bv, Maarssen. The solvents: ethanol, methanol and DMSO 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). D-Glucose and HEPES were procured from ICN Biomedicals, Inc. (Eschwege, Germany). Low gelling temperature agarose, pregnenolone-16α carbonitrile and budesonide 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), 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. ATP Bioluminescence Assay kit CLS II is procured from Roche, Mannheim, Germany. All primers were purchased from Sigma Genosys. All reagents and materials used were of the highest purity that was commercially available.

Animals. Male Wistar (HsdCpb:WU) rats weighing about 230 - 250 g were purchased from Harlan (Horst, The Netherlands). Rats were housed in a temperature and humidity controlled room on a 12-h light/dark cycle with food (Harlan chow no 2018, Horst, The Netherlands) and tap water *ad libitum*. The animals were allowed to acclimatize for 7 days before experimentation. The experimental protocols were approved by the Animal Ethical Committee of the University of Groningen.

Excision of rat intestine and liver. Under isoflurane/O_2/N_2O anaesthesia, the small intestine, colon and liver were excised from the rat. Small intestine and colon were immediately placed into ice-cold carbogenated Krebs-Henseleit buffer, supplemented with 10 mM HEPES, 25 mM sodium bicarbonate and 25 mM D-Glucose, pH 7.4 (KHB) and stored on ice until the preparation of slices. Livers were stored in ice-cold University of Wisconsin solution (UW) until slicing.

Human liver and ileum Tissues. Pieces of human liver tissue were obtained from patients undergoing partial hepactectomy for the removal of carcinoma or from redundant parts of donor livers remaining after split liver transplantation as described previously by Olinga et al. (31). Donor characteristics are given in Table 1. Human ileum was obtained as part of the surgical waste after resection of the ileo-colonic part of the intestine in colon carcinoma patients, donor characteristics are given in Table 2. After surgical resection, the ileum tissue was immediately placed in ice-cold KHB. The research protocols were approved by the Medical Ethical Committee of the University Medical Center, Groningen with informed consent of the patients.

Preparation of rat and human intestinal slices. Rat intestinal slices were prepared as published before (39). In brief, the rat jejunum (at 25-40 cm from the stomach), ileum (5 cm proximal to the ileocecal valve) and colon (large intestine, distal to the ileocecal valve) tissues were separated. The jejunum, ileum and colon were divided into approximately 3-cm segments. The lumen of the segments was flushed with ice-cold KHB that was aerated with carbogen. Thereafter, segments were tied at one end and filled with 3% low gelling agarose solution in saline that was kept at 37° C, then cooled immediately in KHB allowing the agarose to solidify. Subsequently, the agarose filled segments were embedded in agarose solution filled pre-cooled embedding unit (Alabama R&D, Munford, AL, USA). The agarose filled solid embedded intestinal segments were then placed in the pre-cooled Krumdieck tissue slicer (Alabama R&D, Munford, AL, USA) containing carbogenated ice-cold KHB, and precision-cut slices were prepared with a thickness of approximately 200 μm and wet weight of 2-3 mg (without agarose) (cycle speed 40: interrupted mode). Slices were stored in carbogenated ice-cold KHB on ice until the start of the experiment which usually varies between 2 to 3 h after sacrificing the rat.

Human ileum slices were prepared according to the method described for the jejunum (42). In brief, ileum tissue was stripped of the muscular layer and the mucosal

tissue was transferred to carbogenated ice-cold KHB. Mucosal tissue was cut into rectangular pieces of ~ 6-8 mm wide and these were subsequently embedded in low gelling 3% agarose in saline using pre-cooled tissue embedding unit (Alabama R&D, Munford, AL, USA) allowing the agarose solution to solidify. Precision-cut slices of approximately 200-µm thick were prepared as described above for rat intestine.

Preparation of rat and human liver slices. Cylindrical cores of 8 mm were prepared from rat livers and human liver tissue by advancing a sharp rotating metal tube in the liver tissue and were subsequently placed in the pre-cooled Krumdieck tissue slicer. The slicing was performed in carbogenated ice-cold KHB. The thickness of the liver slice was kept at ~ 200-300-µm and a wet weight of 10–12 mg were prepared with the standard settings (cycle speed 40: interrupted mode) of the Krumdieck tissue slicer. Subsequently, slices were stored in ice-cold UW solution on ice prior to the start of the experiment, which usually varies from 1 to 3 h from sacrificing the rat and for human livers 2 to 3 h post surgery.

Induction Studies

Rat and human intestinal slices. Slices were incubated individually in the 12-well, sterile tissue culture plates (Grenier bio-one GmbH, Frickenhausen, Austria) containing 1.3 ml William's medium E supplemented with D-glucose to a 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. Rat intestinal slices were incubated for 12 h because the expression of villin and GAPDH remained unchanged up to 12 h, whereas in pilot experiments, the expression of villin was significantly decreased after 24 h of incubation, indicating loss of epithelial cells. Human ileum slices were incubated for 8 and 24 h, and showed that villin expression remained unchanged up to 24 h. Rat and human intestinal slices were incubated with $1,25(OH)_2D_3$ (final concentrations, 5-100 nM), CDCA (final concentration, 50 µM), DEX (final concentrations, 1-50 µM) and BUD (final concentration, 10 nM). Furthermore, rat intestinal slices were also incubated with PCN (final concentration, 10 µM). All ligands were added as a 100-times concentrated, stock solution in ethanol ($1,25(OH)_2D_3$), methanol (CDCA) and DMSO (DEX/BUD/PCN) and had no or only minor effects on villin expression. Higher concentrations of CDCA (final concentration, 100 µM) significantly reduced villin expression and considered toxic. Control slices were incubated in medium with 1% ethanol, methanol and DMSO without inducers. From a single rat or human tissue sample, six (rat intestine) or three (human intestine) 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 qRT-PCR analysis. Samples were stored in –80° C freezer until RNA isolation. These experiments were replicated in 3-5 rats and 3-5 human ileum donors.

Rat and human liver slices. Slices were incubated individually in 6-well, sterile tissue culture plates (Grenier bio-one GmbH, Frickenhausen, Austria) containing 3.2 ml William's 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. Rat slices were incubated with $1,25(OH)_2D_3$ (final concentrations 10-200 nM), CDCA (final concentrations, 10-100 µM) and DEX (final concentrations, 1-50 µM). Apart from the above inducers, rat liver slices were also incubated with PCN (final concentration, 10 µM) and BUD (final concentrations, 10-100 nM). All inducers were added as a 100-times concentrated stock solution in ethanol (for $1,25(OH)_2D_3$), methanol (for CDCA) and DMSO (for DEX/PCN/BUD). Control rat and human liver slices were incubated in William's medium E with 1% ethanol, methanol, and DMSO, the vehicles. Rat and human liver slices were incubated for 8 h and 24 h, respectively. 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 quantitative real time PCR (qRT-PCR) analysis. Samples were stored in –80° C freezer 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 were isolated by using RNAeasy mini columns from Qiagen according to the manufacturer's instruction. RNA quality and concentrations were determined by measuring the absorbance at 260, 230 nm and 280 nm using a Nanodrop ND100 spectrophotometer (Wilmington, DE, USA). The ratio of absorbance measured at 260 over 280 and 230 over 260 was always above 1.8. About 2 µg of total RNA in 50 µl was reverse transcribed into template cDNA using random primers (0.5 µg / ml), PCR nucleotide mix (10 mM), AMV RT (22 U/µl), RT buffer (10x), $MgCl_2$ (25 mM) and RNAasin (40 U/µl).

qRT-PCR was performed for genes of interest using primer sequences given in Table 3 by two detection systems based on the availability of primer sets; villin and GAPDH were used as house-keeping genes for intestinal epithelial cells and liver cells, respectively, and CYP3A1, CYP3A2, CYP3A9, PXR and FXR were analyzed by the SYBR Green detection system. Primer sequences used for CYP3A1, CYP3A2 and CYP3A9 analysis were identical to those reported earlier by Mahnke et al.,(27). All primer sets were analyzed using BLASTn to ensure primer specificity for the gene of interest **(http://www.ncbi.nlm.nih.gov/BLAST/)**. For qRT-PCR using the SYBR Green detection system ~ 50 ng of cDNA was used in a total reaction mixture of 20 µl of the SYBR Green mixture (Applied Biosystems, Warrington, UK). The PCR conditions are step 1: 95º C for 10 min, and step 2: 40 cycles of 95º C - 15 sec, 56º C - 60 sec, and 72º C - 40 sec, followed by a dissociation stage (at 95º C for 15 sec, at 60º C for 15 sec and at 95º C for 15 sec) to determine the homogeneity of the PCR product. Further, the control consisting of water (with water instead of total mRNA, which has been subjected to reverse transcription

protocol) and the mRNA control (isolated mRNA which has not been subjected to reverse transcription protocol) were used to determine primer dimer formation and contamination of DNA in the isolated samples, respectively. Amplification plots and dissociation curves of the controls did not show any signal and dissociation product, suggesting the lack of primer dimer formation. In addition total RNA from the samples for the preparation of cDNA appeared to be free of DNA contamination. ß-actin and VDR genes were analyzed by Taqman® analysis using primer sequences given in Table 3. For Taqman® analysis ~ 250 ng of cDNA was used in a total reaction mixture of 10 µl Taq Master Mix (2x). The qRT-PCR conditions for Taqman® analysis were: step 1, 95º C for 10 min; step 2, 40 cycles of 95º C for 15 sec and 60º C for 60 sec. All samples were analyzed in duplicates in 384 well plates using ABI7900HT from Applied Biosystems. The comparative threshold cycle (C_T) method was used for relative quantification since C_T was 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 in C_T for gene of interest and the house keeping gene, villin for intestine and GAPDH for liver (ΔC_T). This ΔC_T value of the treated sample 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 $2^{-(\Delta\Delta CT)}$.

ATP and protein content of the human liver slices. Viability of human liver slices during incubation was determined by measuring the ATP contents of the slices according to the method described earlier by de Kanter et al. (3). In brief, control human liver slices were incubated in 3.2 ml of William's medium E, supplemented with D-glucose to a final concentration of 25 mM, gentamicin sulfate (50 µg/ml), and saturated with carbogen, as described in Section 2.7 for 3 and 24 h. At the end of incubation time, three replicate slices were collected individually in 1 ml 70% ethanol (v/v) containing 2 mM EDTA (pH 10.9) and snap-frozen in liquid nitrogen and stored at –80° C freezer until analysis. The samples were disrupted and homogenized by sonication, and ATP extracts were diluted 10 times with 0.1 M Tris HCl containing 2 mM EDTA (pH 7.8) to reduce the ethanol concentration. The ATP content was measured using the ATP Bioluminescence Assay kit CLS II from Roche (Mannheim, Germany) in a 96-well plate Lucy1 luminometer (Anthos, Durham, NC, USA) using a standard ATP- calibration curve.

Protein content of the slices was estimated in three identical, replicate slices which were not used for incubation. The slices were digested with 5 M NaOH and homogenized, and subsequently diluted with water to result in a concentration of 0.1 M NaOH. The protein content of the diluted homogenate was determined by the Bio-Rad protein assay dye reagent method (Bio-Rad, Munich, Germany) using bovine serum albumin (BSA) for the calibration curve. The ATP content of the slice was expressed as pmol/µg of protein.

Statistical analysis. All experiments were performed in 3 to 5 rats and in 4 to 5 human tissue samples. Values were expressed as mean ± S.E.M. All data were analyzed by the paired student's *t*-test or Mann-Whitney *U*-test to detect differences between the means of different treatments. The student's *t*-test was used to analyze the rat data where the error distribution was found to be normal with equal variance except for the CYP3A1 and CYP3A2 genes. Among experiments where non-equal error distribution and high variance (e.g. expression of CYP3A1 and CYP3A2 genes in Wistar rats and CYP3A4 in human tissues due to age and habits) were observed, the non-parametric Mann-Whitney *U*-test was used. 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.

Table 1. Characteristics of human liver donors used: ATP contents after 3 h and 24 h of incubation (each value is a mean ± S.D. of three slices per time point)[a].

Human liver (HL)	Gender	Age	ATP-content (pmol/µg of protein)	
			3 h	24 h
HL – 1[b c]	Female	54	9.2 ± 0.5	10.4 ± 1.5
HL - 2	Not available		4.2 ± 0.9	5.7 ± 1.9
HL – 3[c]	Female	72	3.4 ± 0.8	3.3 ± 1.2
HL - 4	Female	64	7.2 ± 1.2	9.7 ± 1.8
HL - 5[c]	Male	65	12.1 ± 1.2	12.1 ± 1.0
Mean ± S.E.M.			7.2 ± 1.6	8.2 ± 1.6
P - value			0.66	

[a]*Data are expressed as mean ± S.D.*
[b]*Human liver tissue for immunohistochemistry of VDR.*
[c]*Human livers responsive to CYP3A4 induction by 1,25(OH)$_2$D$_3$.*

Table 2. Characteristics of human ileum donors used. The terminal ileum was obtained from colon carcinoma patients as part of tumor resection.

Human ileum (HIL)	Gender	Age	Medical history
HIL - 1	F	85	Colon carcinoma; Coronary disease
HIL - 2	M	60	Colon carcinoma
HIL - 3	F	61	Colon carcinoma
HIL - 4	Not available		
HIL - 5	F	69	Colon carcinoma

Table 3 Oligonucleotides for quantitative real-time PCR, rat and human genes (SYBR Green 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 - CYP3A1	GGAAATTCGATGTGGAGTGC	AGGTTTGCCTTTCTCTTGCC	L24207
r CYP3A2	AGTAGTGACGATTCCAACATAT	TCAGAGGTATCTGTGTTTCCT	XM_573414
r CYP3A9	GGACGATTCTTGCTTACAGG	ATGCTGGTGGGCTTGCCTTC	U46118
r FXR	CCAACCTGGGTTTCTACCC	CACACAGCTCATCCCCTTT	NM_021745
r PXR	GATGATCATGTCTGATGCCGCTG	GAGGTTGGTAGTTCCAGATGCTG	NM_052980
h Villin	CAGCTAGTGAACAAGCCTGTAGAGGAGC	CCACAGAAGTTTGTGCTCATAGGC	NM_007127
h CYP3A4	GCCTGGTGCTCCTCTATCTA	GGCTGTTGACCATCATAAAAG	DQ924960
h VDR	GGAAGTGCAGAGGAAGCGGGAGATG	AGAGCTGGGACAGCTCTAGGGTCAC	NM_000376
r β actin[a]	Assay-by-Design™ FAM labelled, Part number 4331348, (Applied Biosystems).		NM_031144
r VDR[a]	TGACCCCACCTACGCTGACT	CCTTGGAGAATAGCTCCCTGTACT	24873
	Probe - 6FAM - ACTTCCGGCCTCCAGTTCGTATGGAC-TAMRA		
h GAPDH[b]	Assay-by-Design™ ID - Hs99999905_m1		NM_002046
	obe - 6FAM - GCGCCTGGTCACCAGGGCTGCTTTT - NFQ		

r, rat genes and h, human genes, Primer sets for rat[a] Taqman® Gene analysis; Primer sets for human[b] Taqman® Gene analysis.

Results

Expression of nuclear receptors in rat intestine and liver slices. VDR, PXR and FXR mRNA were detected in rat intestine as well as in liver. In rat intestine, PXR, FXR and VDR expression varied along the length of the small intestine and colon. The expression of PXR and VDR relative to villin, which can be considered as representing the expression per epithelial cell, because villin is expressed exclusively in the epithelial cells of the intestine, was 5-fold higher in the colon compared to the jejunum and the ileum (Fig. 1A). The FXR expression relative to villin was 5-fold higher in the ileum compared to the jejunum and was similar to that in the colon (Fig. 1A). For comparison of the expression in liver and intestine, villin cannot be used as housekeeping gene because it is not expressed in hepatocytes. Therefore in this case GAPDH is used as housekeeping gene. In the rat liver, the expression of FXR and PXR relative to GAPDH was significantly higher (2- to 10-fold) compared to that in the small intestine and colon (Fig. 1B). However, the mRNA expression of VDR relative to GAPDH in the rat liver was very low, about 0.1% compared to that in the small intestine and colon but was detectable at an approximate C_T value of 32 to 34 cycles (Fig. 1B).

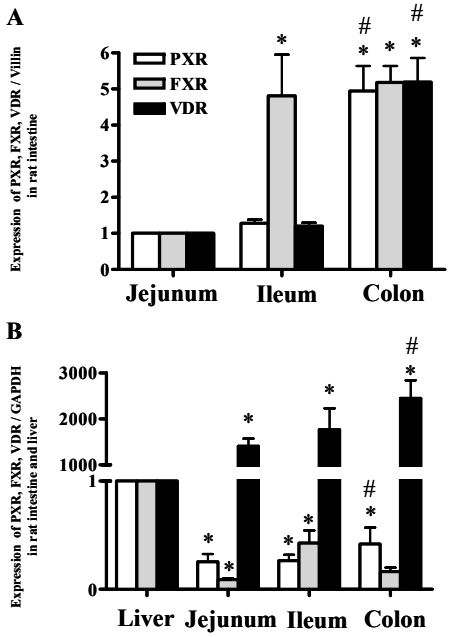

Figure 1. Expression of PXR, FXR and VDR mRNA in rat intestine was normalized to that of villin (A); the value of jejunum/villin was set to 1. The expression of PXR, FXR and VDR mRNA in rat intestine and liver, after normalizing to GAPDH (B), with the liver value set to 1. Results were mean ± S.E.M. of 3 rats. "" denotes P < 0.05, compared to jejunum (A) or liver (B). "#" denotes P < 0.05, compared to ileum (A and B).*

Expression and regulation of CYP3A isozymes in rat intestine slices. Among the CYP3A isozymes in the rat intestine, CYP3A9 was clearly expressed (C_T value ~ 19 to 21) in all segments: the expression of CYP3A9 in rat intestine per enterocyte was in the rank order of colon > jejunum ≥ ileum. CYP3A1 expression was low but detectable (C_T values ≥ 33 for

CYP3A1) in all regions of the intestine. CYP3A2 was barely detectable in the ileum (≥ 35 for CYP3A2) but was undetectable in the jejunum and colon. Because CYP3A1 and CYP3A2 mRNA expression was decreased, whereas that of CYP3A9 expression was moderately elevated during incubation of the slices (data not shown), results on ligand-induced effects were expressed relative to "control" slices incubated with solvent for the same incubation period.

Increasing concentrations of the VDR ligand, $1,25(OH)_2D_3$ strongly induced CYP3A1 mRNA in all regions of the rat intestine (700-fold at 100 nM of $1,25(OH)_2D_3$ in jejunum, 15,000-fold for the ileum, and 1,000-fold for the colon; $P < 0.05$) (Fig. 2A), but the mRNA expression of CYP3A9 remained unchanged (Fig. 2C). In contrast, PCN, DEX and BUD strongly induced CYP3A9 mRNA in the jejunum and ileum, and to a much lesser extent, in the colon (Fig. 2C). PCN and DEX but not BUD induced CYP3A1 in the ileum (Fig. 2A), but had no effect on CYP3A1 in the colon. Although PCN, BUD and DEX induced CYP3A1 mRNA in the jejunum samples, the results failed to reach statistical significance due to the high variation among the data (Fig. 2A). CDCA induced CYP3A1 mRNA only in the ileum and not in the jejunum and colon (Fig. 2A), and failed to affect the expression of CYP3A9 mRNA along the length of the intestine (Fig. 2C). CYP3A2 mRNA, though practically undetectable after incubation with PCN, BUD, DEX and CDCA, was highly induced by $1,25(OH)_2D_3$ in the ileum; however, CYP3A2 remained undetectable in the jejunum and colon for all situations (Fig. 2B).

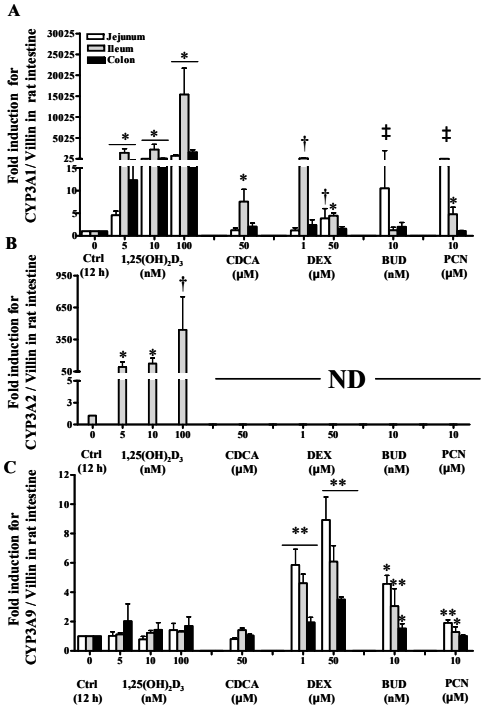

*Figure 2. Slices from rat jejunum, ileum and colon were exposed to $1,25(OH)_2D_3$ (5, 10 and 100 nM), CDCA (50 μM), DEX (1 and 50 μM), BUD (10 nM) and PCN (10 μM) for 12 h, after which total RNA was isolated and mRNA expression of CYP3A1 (A), CYP3A2 (B) and CYP3A9 (C) were evaluated by qRT-PCR. Results were expressed as fold induction after normalizing with villin expression and compared to the control slices of the same segment that was also incubated for 12 h; the control value was set as 1. Results were mean ± S.E.M. of 3-5 rats; in each experiment, 6 slices were incubated per condition. Significant differences towards the control incubations are indicated with *, $P < 0.05$ and **, $P = < 0.01$. "†" denotes induction of CYP3A1 and CYP3A2 in all experiments, but the results failed to reach significance due to the high variation between the experiments, ND – Not detectable; "‡" denotes 1 or 2 out of 3 experiments showed induction.*

Expression and regulation of CYP3A isozymes in rat liver slices. In the rat liver, the expression of CYP3A1 and CYP3A2 was very high compared to that in the intestine, and was detected at a C_T value of 18 to 19, where as CYP3A9 was detected at a C_T value of 22. The expression of CYP3A1, CYP3A2, and CYP3A9 mRNAs was significantly decreased during incubation, but was not further affected by the presence of the solvent vehicle. Distinct from intestinal slices, incubation of rat liver slices with $1,25(OH)_2D_3$ did not change the expression of CYP3A1, CYP3A2 and CYP3A9 (Fig. 3A). DEX induced CYP3A1, CYP3A2 and CP3A9 mRNA expression in rat liver slices in a concentration-dependent manner (Fig. 3C). PCN induced CYP3A1 and CYP3A9 but not CYP3A2 mRNA expression (Fig. 3C). However, BUD induced CYP3A9 expression without affecting those of CYP3A1 and CYP3A2 (Fig. 3C). CDCA significantly decreased the expression of CYP3A1, CYP3A2 and CYP3A9 with increasing concentration (Fig. 3B) to 0.7-fold, 0.5-fold and 0.7-fold, respectively.

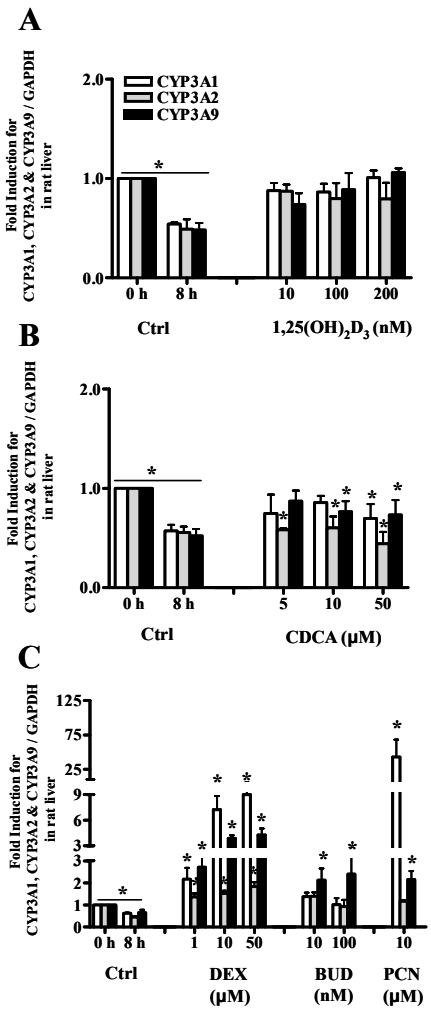

*Figure 3. Slices from rat liver were exposed to $1,25(OH)_2D_3$ (10, 100 and 200 nM) (A), CDCA (50 μM) (B), and DEX (1, 10 and 50 μM), BUD (10 and 100 nM) and PCN (10 μM) (C) for 8 h, after which total RNA was isolated and mRNA expression of CYP3A1 (A), CYP3A2 (B) and CYP3A9 (C) were evaluated by qRT-PCR. Results were expressed as fold-induction, after being normalized to the GAPDH expression, and compared with the control slices that were incubated for 8 h, whose value was set to 1. Results were mean ± S.E.M. of 3-5 rats; in each experiment, 3 slices were incubated per condition. Significant differences towards the control incubations are denoted by *, denoting $P < 0.05$.*

Induction of PXR in rat intestine and liver slices. The expression of PXR, a known GR-responsive gene, was studied in the rat intestinal and liver samples treated with GR (DEX/BUD) and PXR (PCN) ligands. DEX and BUD but not PCN induced PXR expression in all the three regions of the intestine and in the liver (Figs. 4A and 4B). Furthermore, PXR induction by DEX (1 μM) and BUD (10 nM) in the rat colon was lower compared to that in the jejunum and ileum, but the fold-induction at 50 μM DEX, in the jejunum, ileum and colon was comparable (Fig. 4A).

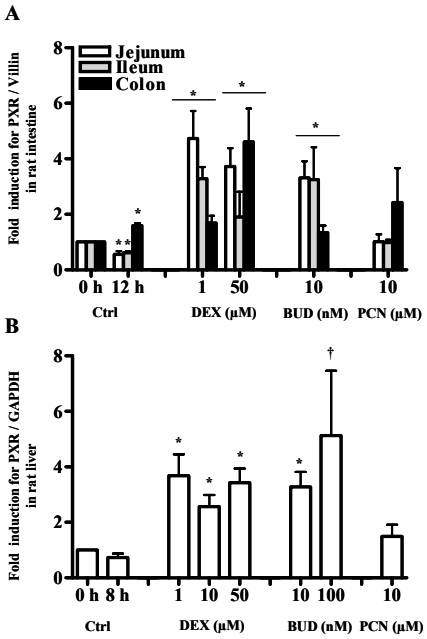

*Figure 4. Slices from rat intestine (jejunum, ileum and colon) were exposed to DEX (1 and 50 μM), BUD (10 nM) and PCN (10 μM) (A) for 12 h. Liver slices were exposed to DEX (1, 10 and 50 μM), BUD (10 and 100 nM) and PCN (10 μM) (B) for 8 h after, which total RNA was isolated and mRNA expression of PXR was evaluated by qRT-PCR. Results were expressed as fold induction after being normalized to the villin for the intestine and GAPDH for liver expression, and compared with the control slices (values set to 1) that were incubated for 12 h and 8 h, respectively,. Results were mean ± S.E.M. of 3-5 rats; in each experiment 6 intestinal and 3 liver slices were incubated per condition. Significant differences towards the control incubations were denoted by *, $P < 0.05$.*

Expression and regulation of CYP3A4 in human ileum liver slices. CYP3A4 mRNA expression was constant up to 8 h of incubation in ileum slices, but decreased to 30-50% by 24 h, with only minor differences between the control and the solvent-treated slices (Fig. 5A). The FXR and PXR expression in human ileum and liver, when expressed relative to GAPDH, was higher in the liver compared to that in the ileum (1.5 to 4 fold); the opposite was observed for the VDR expression, which was significantly higher in the ileum than in the liver. Incubation of ileum slices with increasing concentrations of $1,25(OH)_2D_3$ induced CYP3A4 mRNA expression (Fig. 5B). DEX and BUD but not CDCA also induced CYP3A4 mRNA expression in the ileum slices (Figs. 5C and 5D). In human liver slices, CYP3A4 expression was significantly decreased to 10-20% upon incubation for 24 h in the solvent-treated controls (Fig.6A). $1,25(OH)_2D_3$ induced CYP3A4 in three out of four livers (Fig.6B) (fold induction at 100 and 200 nM were: human liver (HL)1 - 2.66 / 2.29;

HL2 - 2.89 / 0.83; HL3 – 0.33 / 0.25; HL5 – 1.43/1.41). CDCA and DEX induced CYP3A4 significantly in all the 5 human livers studied (Figs. 6C and 6D).

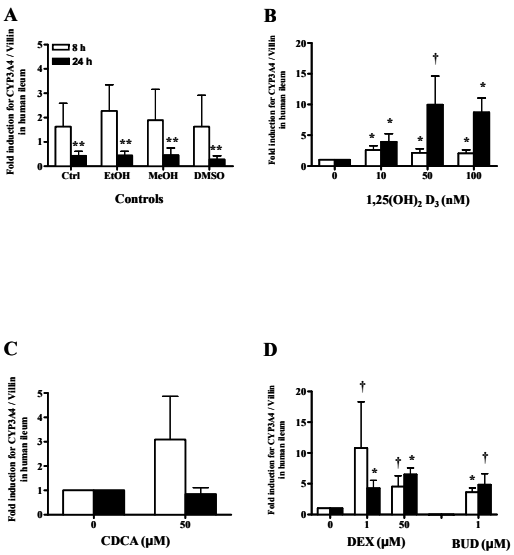

*Figure 5. Slices from human ileum were exposed to control solvents (ethanol, methanol, and DMSO) (A), 1, 25(OH)$_2$ D$_3$ (10, 50 and 100 nM) (B), CDCA (50 μM) (C), DEX (1 and 50 μM) and BUD (10 nM) (D) for 12 h and 24 h, after which total RNA was isolated and mRNA expression of CYP3A4 was evaluated by qRT-PCR. Results were expressed as fold-induction after being normalized to the villin and compared to the control slices (values set as 1) that were incubated for 12 h and 24 h. Results were mean ± S.E.M. of 4-5 human ileum donors; in each experiment, 3 slices were incubated per condition. Significant difference towards the control incubations is denoted by *, P < 0.05 and **, P < 0.01. "†" denotes induction of CYP3A4 in all experiments with high variation between the experiments, and failed to reach statistical significance.*

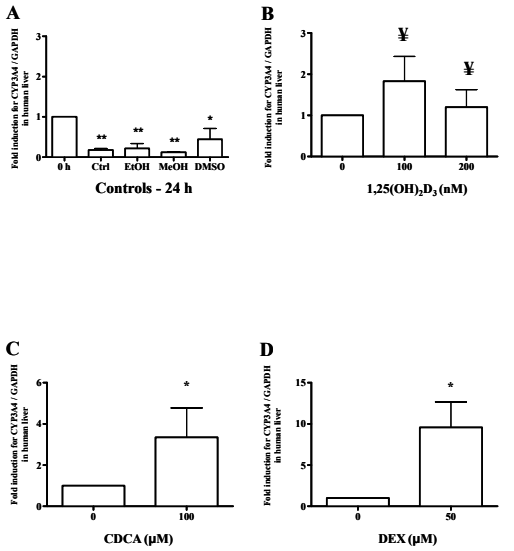

*Figure 6. Slices from human liver were exposed to control solvents (EtOH, MeOH and DMSO) (A), 1, 25(OH)$_2$ D$_3$ (100 and 200 nM) (B), CDCA (100 μM) (C) and DEX (50 μM) (D) for 24 h, after which total RNA was isolated and CYP3A4 mRNA expression was evaluated by qRT-PCR. Results were expressed as fold-induction after being normalized to GAPDH and compared to the control slices (values set as 1) that were incubated for 24 h. Results were mean ± S.E.M. of 5 human liver donors for all ligands except for1,25(OH)$_2$D$_3$ where n = 4; in each experiment 3 slices were incubated per condition. Significant differences towards the control incubations are indicated with *, P < 0.05 and **, P < 0.01. "¥" denotes induction of CYP3A4 in 3 out of 4 human livers, which fails to reach statistical significance.*

The effects of the ligands for VDR, PXR, GR and FXR on the regulation of CYP3A isozymes in rat intestine (jejunum, ileum and colon), rat liver, human ileum and liver are summarized as an overview in Table 4 to facilitate comparison of the effects of the various ligands in the different tissues.

Table 4 Effect of VDR, PXR, FXR and GR ligands on the expression of CYP3A isozymes in rat and human intestine and liver.

Ligands	Nuclear Receptor	Rat												Human	
		CYP3A1				CYP3A2				CYP3A9				CYP3A4	
		Intestine			Liver	Intestine			Liver	Intestine			Liver	IL	Liver
		J	IL	Co		J	IL	Co		J	IL	Co			
$1,25(OH)_2D_3$	VDR	↑	↑	↑	↔	↔	↑	↔	↔	↔	↔	↔	↔	↑	↑[c]
PCN	PXR	↔[a]	↑	↔	↑	Not detectable			↔	↑	↑	↑	↑	Not done	
CDCA	FXR / VDR	↔[a]	↑	↔	↓	Not detectable			↓	↔	↔	↔	↓	↔	↑
BUD	GR	↔[a]	↔	↔	↔	Not detectable			↔	↑	↑	↑	↑	↔	Not done
DEX	PXR / GR	↑[b]	↑	↔	↑	Not detectable			↑	↑	↑	↑	↑	↑	↑

J – Jejunum; IL – Ileum; Co – Colon
↑, induction; ↓, repression; ↔, no induction
[a] *↔, Induction in one out of three experiments*
[b] *↑, Induction with high variation between the experiments*
[c] *↑, Induction in three out of four experiments*

Discussion

In this report, we compared the regulation of CYP3A isozymes by the VDR-specific ligand, $1,25(OH)_2D_3$, in different regions of the intestine and in the liver of the rat and humans with PXR-, GR- and FXR-specific ligands, and investigated whether the changes were related to expression levels of the NRs in the tissues. Most data concerning the regulation of CYP3A isoforms in the rat are restricted to the liver and the small intestine, mostly jejunum, and the data on the comparison of regulation of CYP3A isozymes across the intestinal tract, jejunum, ileum and colon is scarce. *In vivo*, the extent of exposure of the various organs to ligands of the NRs is rarely controlled, and it is difficult to discriminate between the direct and indirect effects of the ligands. In this study, we compared the regulation of gene expression in different segments of the intestine and liver under identical experimental conditions using precision-cut tissue slices (31, 38, 41). Viability of the liver and intestinal slices during incubation was revealed by the stable expression of house-keeping genes, villin as specific gene for enterocytes, and GAPDH for the intestinal and liver tissue, (data not shown). In addition, the ATP content of the human liver slices was assessed as an additional viability marker during incubation; these levels were found to be constant during incubation (Table 1). Furthermore, metabolism in tissue slices is comparable to *in vivo* (12, 40) with adequate expression of transporters and enzymes. Therefore, the uptake and metabolism of ligands is expected to be similar to *in vivo* and to reflect species differences between human and rat. The concentrations of ligands for various nuclear receptors used in this study are similar to those used in earlier studies with metabolically active cells (4, 15), which seem to be adequate to elicit nuclear receptor-specific induction responses, monitored by the induction of signature genes.

In rat intestine, the mRNA expression of PXR, FXR and VDR was found to be present in varying abundances along the length of the small intestine (jejunum and ileum), with the highest expression of all the NRs in the colon. In the rat, the expression of PXR and FXR was 2-10-fold lower in the intestine than in the liver, while in humans, the expression of PXR and FXR was 1.5-4-fold lower in the ileum compared to the liver. Whether this lower liver to intestine ratio of the NRs in humans is significantly different from that in rats could not be concluded from our data, because human samples showed larger inter-individual differences and the liver and intestinal samples were obtained from different patients. The expression of VDR mRNA showed an increasing gradient from rat jejunum to colon, in contrast to the reported gradual decrease of VDR receptor concentration from jejunum to ileum, as determined by the $1,25(OH)_2D_3$ binding assay (5). The VDR expression relative to GAPDH was even higher (up to 2,500-fold) in rat intestine and human ileum compared to rat and human liver, respectively, as reported earlier (1, 32). Immunohistochemical staining showed that the VDR protein was exclusively localized in the bile duct epithelial cells (BECs) in rat livers, whereas in the human livers, not only BEC cells but also hepatocytes contained VDR protein, though to a lower extent (chapter 5), confirming the earlier findings of Gascon-Barre et al. (7). In human livers, the mRNA

expression of VDR showed high inter-individual variations; only three out of four human livers showed detectable VDR expression.

In the rat intestine, the VDR ligand, $1,25(OH)_2D_3$ strongly induced CYP3A1 mRNA along the entire length of the intestine and CYP3A2 only in ileum, but did not affect CYP3A9 expression. The induction of CYP3A1 mRNA by $1,25(OH)_2D_3$ which was found in rat jejunum slices is consistent with earlier *in vivo* report by Xu et al. (45) in Sprague-Dawley rats. We also report on CYP3A1 induction by $1,25(OH)_2D_3$ in ileum and colon slices, with the highest induction occurring in ileum slices compared to the jejunum and colon slices, where induction was similar, despite the highest expression of VDR in colon. Recently, Chow et. al. (2) showed dose-dependent induction of CYP3A1 in the duodenum, jejunum, and ileum and not the colon in the Sprague-Dawley rats *in vivo* after intraperitoneal injections of $1,25(OH)_2D_3$ for 4 days. This is likely explained by lower exposure of the colon than the small intestine to $1,25(OH)_2D_3$ than the small intestine *in vivo*. *In vivo*, CYP3A2 mRNA levels were found to be very low and undetectable, rendering the study of the regulation of CYP3A2 along the length of the rat intestine difficult (2). Recently Xu et al. (45) and Chow et. al. (2) reported that CYP3A2 gene was not responsive to $1,25(OH)_2D_3$ treatment. We also found very low expressions of CYP3A2 mRNA along the length of the intestine of the Wistar rats, and found, surprisingly, that $1,25(OH)_2D_3$ significantly induced CYP3A2 mRNA in the ileum, though not in jejunum and colon slices. Furthermore, CYP3A9 expression was unaffected by $1,25(OH)_2D_3$ along the length of the intestine, as reported by Xu et al. (45) and Chow et. al. (2) in rats *in vivo*. This finding contrasts that of Zierold et al.(47). Our novel observation on the induction of CYP3A2 by $1,25(OH)_2D_3$ in the rat ileum emphasizes the segmental regulation of CYP3A isozymes in the small intestine.

In contrast to the effects of $1,25(OH)_2D_3$ on CYP3A isozymes in rat intestine, the prototypical PXR ligand, PCN, and DEX, which is a GR ligand at low concentrations (< 1 μM) and a PXR ligand at higher concentrations (> 1 μM), induced CYP3A9 mRNA expression in the jejunum, ileum and colon, CYP3A1 in the ileum only, but did not affect the expression of CYP3A2 along the entire length of the intestine. BUD, a specific GR ligand, induced CYP3A9 expression but not CYP3A1 and CYP3A2 in the jejunum, ileum and colon slices. CDCA, the FXR ligand, induced CYP3A1 in the ileum slices but not in jejunum and colon slices. Our results on the induction of CYP3A1 by PXR ligands, PCN and DEX, in rat intestine are consistent with earlier reports on rat jejunum explants (33). Based on the BUD results, a synthetic GR ligand which did not affect CYP3A1 mRNA expression, we conclude that CYP3A1 is not regulated by GR. The induction by DEX at 1 μM, can be explained as a PXR mediated effect, which was further confirmed by the observation that induction of CYP3A1 occurred with PCN, the PXR ligand. The observation on induction of CYP3A9 by PCN, DEX and BUD in rat intestine had not been reported earlier. Our results suggest that apart from PXR, CYP3A9 expression was also regulated by GR. However our data failed to discriminate whether BUD mediated

regulation of CYP3A9 acted via the GRE in the promoter or indirectly via the induction of HNF4α. The induction of PXR by DEX via GR, as reported previously by Huss and Kasper (17), was evident in our studies, since BUD and DEX both induced PXR (Fig. 4). The induction potential of the PXR ligands, PCN and DEX (at 50 μM) on CYP3A1 in the ileum but not in the colon slices did not correlate with the higher expression of PXR in the colon compared to the jejunum.

Although an FXRE has not been identified in the CYP3A1 promoter, the FXR ligand, CDCA, was found to increase CYP3A1 mRNA in the ileum. This observation could be the result of VDR-mediated regulation, since CDCA is also a VDR ligand, albeit of relatively low affinity (28). However, induction of CYP3A1 was not observed in the colon with CDCA despite the high FXR and VDR expression. The possible explanation that CDCA is not efficiently taken up into the colonocytes is in contradiction with our finding that CDCA showed a strong upregulation of the Ostα and Ostβ genes in rat colon slices (20). Among the VDR, FXR, PXR and GR ligands, the VDR ligand, $1,25(OH)_2D_3$, was by far the strongest inducer of CYP3A1 and also induced CYP3A2 in ileum. These results also showed that, although CYP3A1 was upregulated by VDR, PXR and FXR ligands and CYP3A9 by PXR and GR ligands, dramatic differences in the extents of the induction were found in the different segments of the intestine (Table 4). These differences were apparently not related to the differential expression of the respective NRs. The colon, although endowed with an abundance of NRs, exhibited low induction potential of CYP3A isozymes compared to those of the jejunum and ileum.

The VDR-, PXR-, FXR- and GR-dependent regulation of CYP3A1, CYP3A2 and CYP3A9 mRNA in rat liver slices differed dramatically from intestinal slices. The expression of CYP3A1, CYP3A2 and CYP3A9 mRNA was unchanged in liver slices incubated with $1,25(OH)_2D_3$, as found *in vivo* by Xu et al., (45) and Chow et al.,(2). The lack of regulation of CYP3A1 in liver can be explained by the absence of VDR in rat hepatocytes, the major site of the target CYP genes, since VDR is found only in non-parenchymal cells and biliary epithelial cells (7). This was confirmed by immunohistochemisty of rat liver slices in our studies where CYP3A1 was present exclusively in hepatocytes and CYP3A2, mainly in hepatocytes, and expressed at a much lower level in biliary epithelial cells (unpublished observations).

In contrast, PCN and DEX induced the expression of hepatic CYP3A1 and CYP3A9, and BUD induced CYP3A9, whereas CYP3A2 expression was modestly induced only by DEX. These data on the induction of CYP3A1 and CYP3A9 by the PXR ligands, PCN and DEX, agree with earlier reports on Sprague-Dawley rats (16, 27). However, the induction of CYP3A9 by BUD (GR), though suggested by Komori and Oda (22), has not been reported earlier. Induction of CYP3A9 by PCN and DEX in the rat liver and intestine implies that CYP3A9 is likely regulated by PXR via a PXRE. However, it remains to be elucidated whether the inductive effect of BUD on CYP3A9 is directly mediated via GR

and a GRE in the CYP3A9 promoter, or indirectly via upregulation of PXR and HNF4α by BUD. The two stage induction by the GR on the upregulation of CYP via induction of PXR has been suggested for CYP3A1/23 (17), and is a likely possibility since PXR induction was also observed with GR ligands (Fig. 4).

Unlike the induction of CYP3A1 observed in the ileum, CDCA, an FXR ligand, showed repression of CYP3A1, CYP3A2 and CYP3A9 in rat liver slices. This might be due to the CDCA mediated repression of PXR (chapter 4), which in turn, affects the basal expression of CYP3A isozymes in rat liver. These results are in stark contrast to the mouse studies of Jung et al. (19), where *Cyp3a11* and *pxr* were positively regulated by FXR in mice that were treated with FXR agonists, cholic acid and GW4064, suggesting species difference in the regulation of these genes.

In the human ileum, CYP3A4 was significantly induced by VDR, PXR and GR ligands (Figs. 6B and 6D). Although the regulation of CYP3A4 *in vivo* by PXR and GR ligands in the human intestine is well known, we are the first to show the regulation of CYP3A4 in human intestinal tissue by the VDR ligand, $1,25(OH)_2D_3$. These observations agree with findings in cultured monolayers such as Caco-2 and LS180 cells (6, 34). Also in human liver slices, CYP3A4 is upregulated by $1,25(OH)_2D_3$, but only in those three liver samples that showed expression of VDR, as reported for human hepatocytes (4). For the liver that did not express VDR, no upregulation of CYP3A4 by $1,25(OH)_2D_3$ was found. This variation between samples was not due to viability differences because human liver slices treated with the PXR/GR ligand, DEX induced CYP3A4 in all the experiments, observations that were in agreement with earlier report (23). CDCA induced expression of CYP3A4 in human liver, confirming the FXR-dependent regulation in earlier reports by Gnerre et al. (8). But this response was not observed in the ileum. It appears that all NRs: VDR, PXR, GR and FXR, are able to induce CYP3A4 in the human liver.

In summary, studies in tissue slices showed that the overall effects of ligands for NRs on regulation of CYP3A isozymes differed in different regions of the rat intestine and liver, and human ileum and liver slices, despite the incubation was conducted under identical circumstances. This difference appears not to be directly related to the different expression levels of the nuclear receptors involved. In the rat intestine, CYP3A1 expression is very sensitive to the VDR ligand, and to a lesser extent, to PXR and GR ligands, whereas CYP3A2 expression is exclusively regulated by the VDR. CYP3A9 expression both in the liver and in all regions of the intestine appears to be mainly regulated by PXR and GR but not by VDR. In human tissue, however, CYP3A4 in ileum and liver was upregulated by PXR, VDR and GR ligands. By contrast, CDCA elicited varying effects, ranging from decreased expression in rat liver, lack of effect in human ileum, and increased expression in rat ileum and human liver, effects that are not explained by the expression of FXR. Our results suggest that prediction of the inducing potential of drugs should not rely strictly on whether or not the drug under study is a ligand for a certain NR

and the expression levels of this NR in the target organ. Uptake, metabolism and excretion of the ligand as well as the availability of co-activators or repressors in the specific tissue and species may play a decisive role.

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Chapter3

The Role of Lithocholic Acid in the Regulation of Bile acid Detoxification, Synthesis and Transport proteins in Rat and human Intestine and Liver Slices

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Abstract

The effects of lithocholic acid (LCA), a common ligand of VDR, FXR and PXR, on the regulation of proteins involved in bile acid metabolism (CYP3A isozymes), synthesis (CYP7A1) and transport (MRP3, MRP2, BSEP, NTCP), as well as the nuclear receptors (FXR, PXR, LXRα, HNF1α, HNF4α and SHP) that are involved in their regulation, were studied in the rat and human precision-cut intestine and liver slices at the level of mRNA as quantified by qRT-PCR. The LCA effects were compared to those from specific PXR, FXR and VDR ligands. LCA induced CYP3A1 and CYP3A9 in the rat jejunum, ileum and colon, CYP3A2 only in the ileum, and CYP3A9 expression in the liver only. LCA induced CYP3A4 in the human ileum and not in liver. Based on the data obtained with specific VDR, PXR and FXR ligands, we conclude that LCA induced the expression of detoxification enzymes via by the VDR (CYP3A1, CYP3A2 and CYP3A4) and PXR (CYP3A9) both in the rat and the human intestine. The expression of rMRP2 was induced in the colon but not in the jejunum and ileum that for rMRP3 was not affected for segments of the intestine in the rat, whereas in human ileum slices, LCA induced hMRP3 and hMRP2 expression. In the rat liver, LCA decreased rCYP7A1, rLXRα and rHNF4α expression, and induced rSHP expression but did not affect rBSEP and rNTCP expression, whereas in the human liver, a small but significant decrease was found for hHNF1α expression. These data suggests profound species differences in the effects of LCA on bile acid transport, synthesis and detoxification. The LCA-induced altered enzymes and transporter expressions in the intestine may have consequences for drug disposition in conditions with elevated LCA levels such as cholestasis.

Keywords: Cytochrome P450, induction, intestinal slices, liver slices, lithocholic acid.

Introduction

Lithocholic acid (LCA) is a toxic secondary monohydroxy bile acid (BA) formed by the bacterial biotransformation (7α-dehydroxylation) of the primary BA, chenodeoxycholic acid (CDCA) in the terminal part of the small intestine (12, 24). LCA is passively absorbed by the intestinal mucosal cells (35, 51, 55), metabolized and excreted either back into the intestinal lumen or into the portal blood. LCA is reported to be carcinogenic in the intestine and cholestatic in the liver of animals and man (Javitt, 1966; Fisher et al., 1971; Narisawa et al., 1974). It is metabolized by cytochrome P450 (CYP) enzymes in the intestine and the liver of humans (CYP3A4) and rats (CYP3A1, CYP3A2, CYP3A9, CYP2C6, CYP2C11 and CYP2D1) to 6α- and 6β-hydroxy metabolites, respectively (3, 17). Recently, 3-keto-5β-cholanic acid (3KCA) was identified as the major LCA metabolite with human recombinant CYP3A4 isozyme (7). Moreover LCA is conjugated by sulfotransferase into sulfolithocholic acid (27).

Because LCA is predominantly formed in the terminal part of the small intestine and the ileal mucosal cells are exposed to very high concentrations of LCA. Nevertheless, most of the studies on LCA biotransformation were performed in liver microsomes of different species, and data are not available for intestine. Furthermore, the LCA-mediated regulation of the CYP isozymes involved in its metabolism is incompletely understood in the intestine and the liver. Makishima et al. (41), Wolf et al. (59) and Staudinger et al. (53) independently showed that LCA and its metabolite, 3KCA can bind and transactivate the vitamin D receptor (VDR), the pregnane X receptor (PXR) and the farnesoid X receptor (FXR) upon binding of these nuclear receptors (NR's) to their respective response elements in the promoters of the target genes as a heterodimer with retinoic acid X receptor α (RXRα). Hence, it is hypothesized that LCA and its metabolites may coordinately regulate bile acid detoxification, synthesis and transport proteins in the intestine and the liver.

In addition to the well known involvement of the PXR and glucocorticoid receptor (GR) in the regulation of CYP3A isoforms, 1α,25-dihydroxy vitamin D_3 ($1,25(OH)_2D_3$), a VDR ligand was recently reported to regulate the CYP3A isozymes involved in the detoxification of LCA in human and rat intestinal cell lines and human foetal intestine explants (9, 20, 50, 56). Recently, we observed $1,25(OH)_2D_3$-dependent regulation of the rat CYP3A isozymes in the small intestine, colon and liver, and CYP3A4 in the human ileum and liver using precision-cut slices (33). Our results were consistent with the induction of CYP3A1 in duodenum, jejunum and ileum of Sprague-Dawley rats treated with intraperitoneal (ip) injection of $1,25(OH)_2D_3$ for four days (11, 60).

Bile acids (BAs) are synthesized from cholesterol in the liver by the sequential activity of 12 enzymes in which CYP7A1 catalyzes the first rate limiting step (49). Upon amidation with glycine and taurine, BA's are secreted into the intestine via the bile and reabsorbed in the terminal part of the small intestine and transported back to the liver, a

process known as the enterohepatic cycle (28). During the enterohepatic cycle, BAs and their conjugates are transported across the membranes of hepatocytes and enterocytes by members of the ATP binding cassette (ABC) transporter family, multidrug resistance-associated protein (MRP) 2, 3 and 4, bile salt export pump (BSEP; ABCB11), apical sodium dependent bile acid transporter (ASBT; SLC10A2), sodium dependent taurocholate co-transporting polypeptide (NTCP; SLC10A1) and OSTα-OSTβ (2, 16, 25, 26, 46, 52, 54, 61). Together, these transporters contribute to the maintenance of the bile acid pool *in vivo*. BAs synthesis and transport are regulated at the transcriptional level by various nuclear receptors such as PXR, FXR, CAR and VDR (2, 19, 29, 32, 37, 45).

The effects of LCA on the regulation of enzymes and transporters had been studied in the liver of rodents after LCA administration (6). In man, however, no direct data is available but the role of LCA was indirectly studied in human cholestatic livers (62, 63). In these *in vivo* studies, it is difficult to control the exposure of LCA to the different organs and it is not possible to discriminate between direct effects of LCA and indirect effects of cholestasis or other potential confounding factors. Also, no data is available on the role of LCA in the regulation of enzymes and transporters involved in BA homeostasis for the human and rat intestine. Therefore we conducted a systematic study to investigate the direct effects of LCA on the regulation of BA detoxification enzymes and transporters in the rat and human intestine and liver, the BA synthesis enzyme, CYP7A1, in rat and human livers, and the nuclear receptors/transcription factors involved in the regulation of these proteins at the level of mRNA by exposing precision-cut organ slices to different concentrations of LCA. Previously, this *in vitro* model was shown to be a valuable model to study regulation of genes of interest by ligands for several NR in liver (23, 30, 47) and intestine (33, 34, 43, 57) under identical conditions. Further, we compared the LCA mediated effects with those induced from other, specific nuclear receptor ligands, CDCA for FXR, PCN for PXR, DEX for GR and PXR, and budesonide (BUD) for GR.

Materials and methods

Chemicals and Reagents. Lithocholic acid and chenodeoxycholic acid were purchased from Calbiochem, San Diego, CA, dexamethasone was from Genfarma bv, Maarssen. Pregnenolone-16α carbonitrile, budesonide and the solvents; ethanol, methanol and dimethylsulfoxide 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, HEPES were procured from ICN Biomedicals, Inc. (Eschwege, Germany). University of Wisconsin organ preservation solution (UW) was obtained from Du Pont Critical Care, Waukegab, Illinois, USA. Low gelling temperature agarose was 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), PCR nucleotide mix (10 mM), AMV RT (22 U/μl) and RNasin (40 U/μl); were purchased from Promega

Corporation, Madison WI, USA. SYBR Green and Taqman Master Mixes (2x) were purchased from Applied Biosystems, Warrington, UK, Abgene Westbrug and Eurogentech, respectively. All primers were purchased from Sigma-Genosys by order on demand. All reagents and materials used were of the highest purity that was commercially available.

Animals. Male Wistar (HsdCpb:WU) rats weighing about 230-250 g were purchased from Harlan (Horst, The Netherlands) and were allowed to acclimatize for 7 days before experimentation. Rats were housed in a temperature and humidity controlled room on a 12 h light/dark cycle with food (Harlan chow no. 2018, Horst, The Netherlands) and tap water ad libitum. The experimental protocols were approved by the Animal Ethical Committee of the University of Groningen.

Human Liver and Ileum Tissue. Pieces of human liver tissue were obtained from patients undergoing partial hepactectomy for the removal of carcinoma or from redundant parts of donor livers remaining after split liver transplantation, as described previously (47). After surgical resection, human liver tissue was immediately placed in ice-cold University of Wisconsin (UW) organ storage solution. Human liver donor (n=5) characteristics are as reported earlier (33). Further, two additional human livers were used for LCA experiments and their donor characteristics (human livers HL6 and HL7) are illustrated in table 3. The human ileum was obtained as part of the surgical waste after resection of the ileocolonic part of the intestine in colon carcinoma patients; the donor characteristics were identical to those reported earlier (33). After surgical resection, the ileum tissue was immediately placed in ice-cold Krebs-Henseleit buffer supplemented with 10 mM HEPES, 25 mM sodium bicarbonate and 25 mM D-glucose, pH 7.4 (KHB). The research protocols were approved by the Medical Ethical Committee of the University Medical Center, Groningen, with informed consent of the patients.

Preparation of Rat and Human Intestinal Slices. Rat intestinal slices were prepared as published before (33, 57). In brief, rat jejunum (at 25-40 cm from the stomach), ileum (5 cm proximal to the ileocecal valve) and colon (large intestine, distal to the ileocecal valve) tissues were luminally flushed with carbogenated ice-cold KHB, filled and embedded with 3% low gelling agarose using a pre-cooled embedding unit (Alabama R&D, Munford, AL USA). The agarose filled, solid embedded, intestinal segments were then placed in the pre-cooled Krumdieck tissue slicer (Alabama R&D, Munford, AL USA) containing carbogenated ice-cold KHB and precision-cut slices were prepared with a thickness of approximately 200-µm and wet weight of 2-3 mg (without agarose) with standard settings (cycle speed 40: interrupted mode). Slices were stored in carbogenated ice-cold KHB on ice until the start of the experiment which usually varies between 2 to 3 h after sacrificing the rat. Human ileum slices were prepared according to the method described previously for human intestinal slices (33, 58). In brief, ileum tissue was stripped of the muscular layer and the mucosal tissue was embedded in low gelling 3% agarose in saline. Precision-cut slices of approximately 200-µm thick were prepared as described above for rat intestine.

Preparation of Rat and Human Liver Slices. Human and rat liver slices were prepared as described earlier (33), from cylindrical cores of liver tissue (8 mm) using the Krumdieck tissue slicer (cycle speed 40: interrupted mode). The thickness of the liver slice was kept at ~ 200-300-µm (wet weight of 10-12 mg). The slices were stored in ice-cold UW solution on ice prior to the start of the experiment, which usually varies from 1-3 h from sacrificing the rat and for human livers 2-3 h post surgery.

Induction Studies

Rat and Human Intestinal Slices. Precision-cut slices 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 to a final concentration of 25 mM, gentamicin sulfate, 50 µg/ml amphotericin/fungizone, 250 µg/ml and saturated with carbogen at 37° C, continuously gassed with carbogen and shaken at 80 rpm. Rat intestinal slices were incubated with LCA (final concentrations 5 and 10 µM), CDCA (final concentration, 50 µM), DEX (final concentrations, 1 and 50 µM) and PCN (final concentration, 10 µM) added as a 100-times concentrated stock solution in methanol (LCA and CDCA) and DMSO (DEX and PCN), and incubated for 12 h. Human ileum slices were incubated with LCA (final concentration 10 µM) and CDCA (final concentration 50 µM) added as a 100-times concentrated stock solution in methanol and incubated for 8 and 24 h. Control slices from rat intestine and human ileum were incubated in supplemented Williams medium E with 1% methanol or DMSO without inducers. From a single rat or human tissue sample, six (rat intestine) or three (human intestine) 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 in -80° C freezer until RNA isolation. These experiments were replicated in 3-5 rats and with human ileum samples from 3-5 donors.

Rat and Human Liver Slices. Rat and human liver slices were incubated individually in 6-well sterile 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 at 37° C, continuously gassed with carbogen and shaken at 80 rpm. Rat liver slices were incubated with LCA (final concentration, 50 µM), DEX (final concentration, 50 µM), BUD (final concentrations, 10 and 100 nM) and PCN (final concentration, 10 µM) added as 100-times concentrated stock solution in methanol (for LCA and CDCA) and DMSO (for DEX, BUD and PCN). Control slices were incubated in supplemented Williams medium E with 1% methanol and DMSO without inducers for 8 and 24 h. Human liver slices were incubated with LCA and DEX (final concentration, 50 µM) added as 100-times concentrated stock solution in methanol and DMSO, respectively. Control slices were

incubated in supplemented Williams medium E with 1% methanol or DMSO without inducers for 24 h. 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 in -80° C freezer until RNA isolation. These experiments were replicated in 3-5 rats and with liver samples from 4-5 human liver donors.

RNA Isolation and qRT-PCR. Total RNA from the rat and human intestine and liver samples was isolated with RNAeasy mini columns from Qiagen by following manufacturer's instruction. RNA concentration and quality were determined by measuring the absorbance at 260, 230 and 280 nm using a Nanodrop ND100 spectrophotometer (Wilmington, DE USA). The ratio of absorbance measured at 260/280 and 260/230 was always above 1.8 for all the samples. The total RNA (2 µg/50 µl) was reverse-transcribed into template cDNA with Promega reverse-transcription kit from Promega Corporation, Madison WI, USA according to the earlier published method (33).

qRT-PCR was performed for the rat and human genes using primer sequences listed in table 1 and 2, respectively, using SYBR Green detection system as reported earlier by Khan et al. (33). Primer sequences used for CYP3A1, CYP3A2 and CYP3A9 analysis were as reported earlier by Mahnke et al.,(40). All primer sets were analyzed using BLASTn to ensure primer specificity for the gene of interest (http://www.ncbi.nlm.nih.gov/BLAST/). Furthermore appropriate controls were analyzed for all the primer sets to determine dimer formation of the primer and homogeneity of the PCR products. The comparative threshold cycle (C_T) method was used for relative quantification of the mRNA. 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 in C_T for gene of interest and the reference gene (villin for intestine and GAPDH for liver) (ΔC_T), which was compared to the ΔC_T of the corresponding solvent control ($\Delta\Delta C_T$). Data are expressed as fold induction or repression of the gene of interest according to the formula $2^{-(\Delta\Delta CT)}$. No significant differences were observed in the expression of genes of interest between control incubations with and without the solvents (methanol and DMSO), therefore all control incubation data was analyzed as one experimental group.

Statistical analysis. All experiments were performed in 3-5 rats and in 5-7 human tissue samples. All values were expressed as mean ± S.E.M. All data were analyzed by the paired student's *t*-test or Mann-Whitney *U*-test to detect the effect of the exposure to the ligands. The student's *t*-test was used to analyze the rat data where the error distribution was found to be normal with equal variance. The non parametric Mann-Whitney *U*-test was used for experiments where non-equal error distribution and high variance (e.g. expression of CYP3A1 and CYP3A2 genes in Wistar rats and all genes in human tissues)

were observed. Statistical analysis was performed on fold induction as well as on $\Delta\Delta C_T$ with similar results. A P value < 0.05 was considered as significant.

Table 1 *Oligonucleotides for quantitative Real-Time PCR, rat genes (SYBR Green analysis)*

Gene	Forward primer (5' – 3')	Reverse primer (5' – 3')
r Villin	GCTCTTTGAGTGCTCCAACC	GGGGTGGGTCTTGAGGTATT
r GAPDH	CTGTGGTCATGAGCCCCTCC	CGCTGGTGCTGAGTATGTCG
r - CYP3A1	GGAAATTCGATGTGGAGTGC	AGGTTTGCCTTTCTCTTGCC
r CYP3A2	AGTAGTGACGATTCCAACATAT	TCAGAGGTATCTGTGTTTCCT
r CYP3A9	GGACGATTCTTGCTTACAGG	ATGCTGGTGGGCTTGCCTTC
r CYP7A1	CTGTCATACCACAAAGTCTTATGTCA	ATGCTTCTGTGTCCAAATGCC
r BSEP	TGGAAAGGAATGGTGATGGG	CAGAAGGCCAGTGCATAACAGA
r NTCP	CTCCTCTACATGATTTTCCAGCTTG	CGTCGACGTTCGTTCCTTTTCTTG
r MRP2	CTGGTGTGGATTCCCTTGG	CAAAACCAGGAGCCATGTGC
r MRP3	ACACCGAGCCAGCCATATAC	TCAGCTTCACATTGCCTGTC
r SHP	CTATTCTGTATGCACTTCTGAGCCC	GGCAGTGGCTGTGAGATGC
r HNF1α	CTCCTCGGTACTGCAAGAAACC	TTGTCACCCCAGCTTAAGACTCT
r HNF4α	CCAGCCTACACCACCCTGGAGTT	TTCCTCACGCTCCTCCTGAA
r LXRα	TGCAGGACCAGCTCCAAGTA	GAATGGACGCTGCTCAAGTC
r LRH-1	GCTGCCCTGCTGGACTACAC	TGTAGGGCACATCCCCATTC
r PXR	GATGATCATGTCTGATGCCGCTG	GAGGTTGGTAGTTCCAGATGCTG
r FXR	CCAACCTGGGTTTCTACCC	CACACAGCTCATCCCCTTT

r, rat

Table 2 *Oligonucleotides for quantitative Real-Time PCR, human genes (SYBR Green and Taqman® analysis)*

Gene	Forward primer (5' – 3')	Reverse primer (5' – 3')
h Villin	CAGCTAGTGAACAAGCCTGTAGAGGAGC	CCACAGAAGTTTGTGCTCATAGGC
h GAPDH	ACCCAGAAGACTGTGGATGG	TCTAGACGGCAGGTCAGGTC
h CYP3A4	GCCTGGTGCTCCTCTATCTA	GGCTGTTGACCATCATAAAAG
h CYP7A1	GCTGTTGTCTATGGCTTATTCTT	GCCCAGGTATGGAATTAATCCA
h BSEP	CAGTTCCCTCAACCAGAACAT	TTTGATCATTTCGCTCTCGATG
h NTCP	CTCAAATCCAAACGGCCACAAT	CACACTGCACAAGAGAATGATGATC
h MRP2	CGGACAGCATCATGGCTTCT	ACTCCTTCCTTGGCCAAGTTG
h MRP3	GTCCGCAGAATGGACTTGAT	TCACCACTTGGGGATCATTT
h SHP	TGAAAGGGACCATCCTCTTCA	CAATGTGGGAGGCGGCT
h HNF1α	CAGAAAGCCGTGGTGGAGAC	GACTTGACCATCTTCGCCACA
h HNF4α	CCTGGAATTTGAGAATGTGCAG	AGGTTGGTGCCTTCTGATGG
h LXRα	CCCTTCAGAACCCACAGAGATC	GCTCGTTCCCCAGCATTTT
h PXR	CCCAGCCTGCTCATAGGTTC	GGGTGTGCTGAGCATTGATG
h FXR	AGAGATGGGAATGTTGGCTGA	GCATGCTGCTTCACATTTTTTC
h GAPDH	Assay-on-Demand™ ID - Hs99999905_m1	
	Probe sequence (5'FAM -3' NFQ) - GCGCCTGGTCACCAGGGCTGCTTTT	

h, human

Results

Regulation of CYP3A isozymes by LCA in rat intestine and liver slices. Incubation of rat intestinal slices (jejunum, ileum and colon) with LCA induced CYP3A1 expression along the length of the intestine, showing a very high induction in the jejunum (400-fold at 10 μM of LCA; $P < 0.05$) and ileum (550-fold at 10 μM of LCA; $P < 0.05$), and a moderate induction in the colon (3.5-fold at 10 μM of LCA; $P < 0.05$) (Fig. 1A). LCA induced CYP3A2 in the rat ileum slices but not in the jejunum and colon slices (Fig. 1B). Induction of CYP3A2 in the rat ileum slices by LCA was found to be significant at 5 μM (5-40-fold;

$P < 0.05$). At 10 μM of LCA, CYP3A2 induction was even higher but showed a high variation among the rats ranging from 6- to 124-fold and therefore failed to reach statistical significance. Furthermore, LCA induced CYP3A9 in jejunum, ileum and colon slices (2-3-fold; $P < 0.001$) with a slightly higher effect in the colon slices (Fig. 1C).

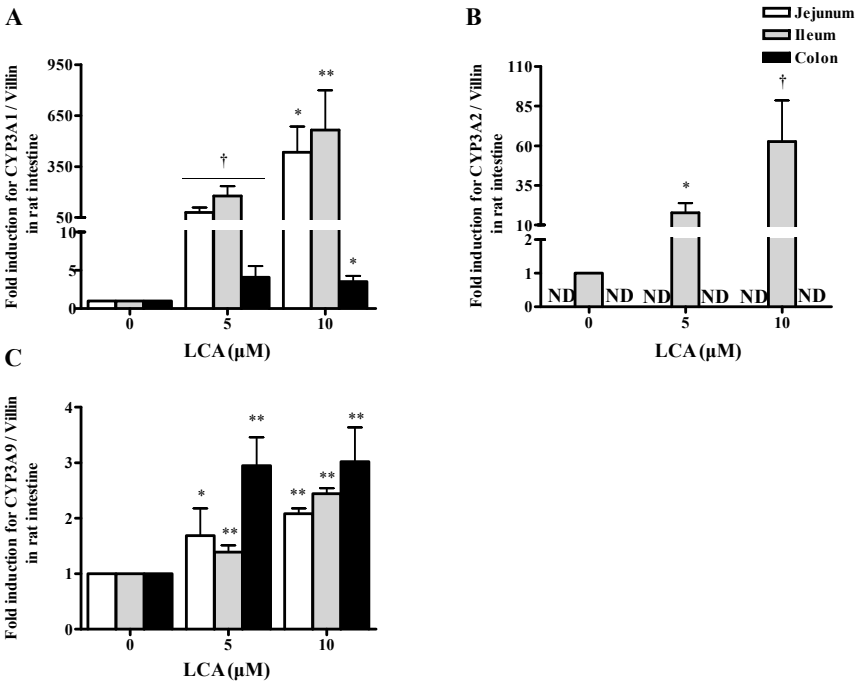

*Figure 1. Rat jejunum, ileum and colon tissue-slices were exposed to LCA (5 and 10 μM) for 12 h after which total RNA was isolated and mRNA expression of CYP3A1 (A), CYP3A2 (B) and CYP3A9 (C) were evaluated by qRT-PCR. After normalizing for villin expression the results were compared to the 12 h incubated control slices of the same segment. Results showed mean ± S.E.M. of 3-5 rats; in each experiment, 6 slices were incubated per condition. Significant differences towards the control incubations are indicated with * $P < 0.05$, and ** $P < 0.001$. "†"denotes induction of CYP3A1 and CYP3A2 in all experiments, but failed to reach statistical significance due to high variation between the experiments, ND-not detectable.*

In contrast to the intestinal slices, the expression of CYP3A1, CYP3A2 and CYP3A9 mRNA in the rat liver slices was not affected by LCA during 8 h of incubation. However, when liver slices were exposed to LCA for 24 h, CYP3A9 expression was induced by 2-fold, whereas CYP3A1 and CYP3A2 expression remained unaltered (Fig. 2). As expected, the PXR ligands dexamethasone (DEX) and pregnenolone-16α carbonitrile (PCN) significantly induced the expression of CYP3A1 (100-fold) and CYP3A9 (5-8-fold; $P < 0.001$), but not CYP3A2 (Fig. 2). Budesonide (BUD), a synthetic GR ligand significantly induced CYP3A9 expression (2-3-fold; $P < 0.001$) and decreased CYP3A2 expression, but did not influence CYP3A1 expression (Fig. 2). These results with PCN, DEX and BUD show that the PXR and GR mediated pathways are intact in rat liver slices.

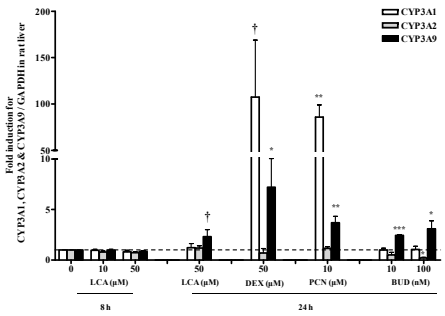

*Figure 2. Rat liver slices were exposed to LCA (10 to 50 μM) for 8 h and 24 h; and with DEX (50 μM), PCN (10 μM) and BUD (10 and 100 nM) for 24 h, after which total RNA was isolated and mRNA expression of CYP3A1, CYP3A2 and CYP3A9 was evaluated by qRT-PCR. After normalizing for GAPDH expression, the results were expressed as fold-induction and compared with the 8 h and 24 h incubated control slices. Results showed mean ± S.E.M. of 3-5 rats; 3 slices were incubated per condition in each experiment. Significant differences towards the control incubations are indicated with * P < 0.05, ** P < 0.001 and *** P < 0.0001. "†"denotes induction of CYP3A9 in all experiments, but failed to reach statistical significance due to high variation between the experiments.*

Expression and regulation of CYP3A4 in human ileum and liver slices. CYP3A4 expression was stable during control incubation of the human ileum slices for 8 h, but was decreased in human ileum and liver slices after 24 h of incubation (Fig. 3A). Incubation of human ileum slices with LCA (10 μM) significantly induced CYP3A4 expression (9- and 5-fold induction during 8 h and 24 h, respectively; $P < 0.05$) (Fig. 3B). Incubation of human liver slices with LCA (50 μM) induced CYP3A4 mRNA expression in four out of seven livers, and slightly reduced it in the other three livers (Fig. 3B and table 3).

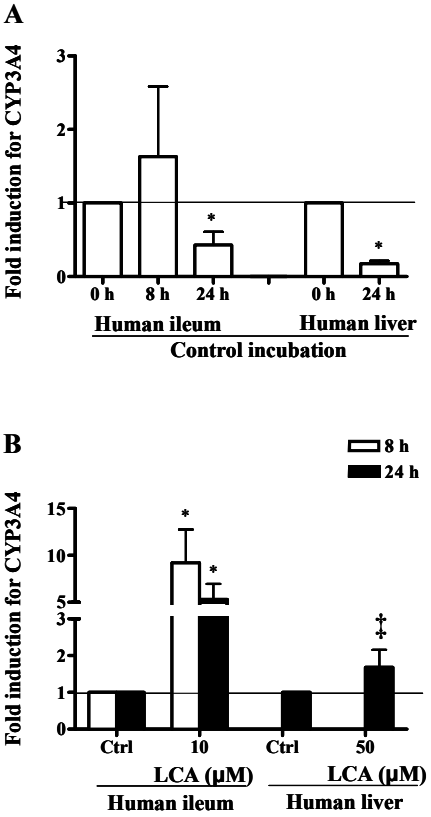

*Figure 3. Slices from human ileum and liver were exposed to control incubation (A), LCA, 10 μM and 50 μM (B), respectively for 8 h and 24 h, after which total RNA was isolated and mRNA expression of CYP3A4 was evaluated by qRT-PCR. Results were expressed as fold-induction after normalizing with villin for ileum and GAPDH for liver, 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 4 human ileum and 7 liver donors; in each experiment 3 ileum and liver slices were incubated per condition. Significant differences towards the control incubations are indicated with * P < 0.05. "‡"denotes induction of CYP3A4 in 4 out of 7 experiments.*

Expression and regulation of rMRP2 and rMRP3 in rat intestine slices. In rat intestine, rMRP2 and rMRP3 transporters are expressed in reciprocal gradients along the length of the intestine with rMRP2 mRNA expression showing a decreasing gradient from the jejunum towards the colon, and rMRP3 expression showed an increasing gradient from the jejunum to the colon (Fig. 4). During control incubations of rat jejunum, ileum and colon slices in Williams Medium E without ligands, rMRP2 expression was significantly increased in jejunum and ileum, but decreased in colon slices (Fig. 5A). rMRP3 expression was significantly increased in jejunum, ileum as well as in colon slices during control incubation (Fig. 5C). LCA induced the expression of rMRP2 compared to the control incubations in colon slices but not in jejunum and ileum slices (Fig. 5B). LCA did not affect the rMRP3 expression along the length of the intestine (Fig. 5D). In contrast, CDCA significantly decreased the expression of rMRP2 in jejunum and ileum slices but induced rMRP2 in colon slices (Figs. 5B and D), whereas CDCA increased the rMRP3 expression in ileum slices only but did not affect the rMRP3 expression in jejunum or colon (Figs. 5B and D). DEX significantly induced the rMRP2 expression in the jejunum and colon slices and decreased the expression of rMRP3 in jejunum and ileum but not in colon slices (Fig. 5B and D). Similar to DEX, PCN significantly induced the rMRP2 expression in the jejunum and colon slices (Fig. 5B) but PCN did not affect the rMRP3 expression along the length of the rat intestine (Fig. 5D).

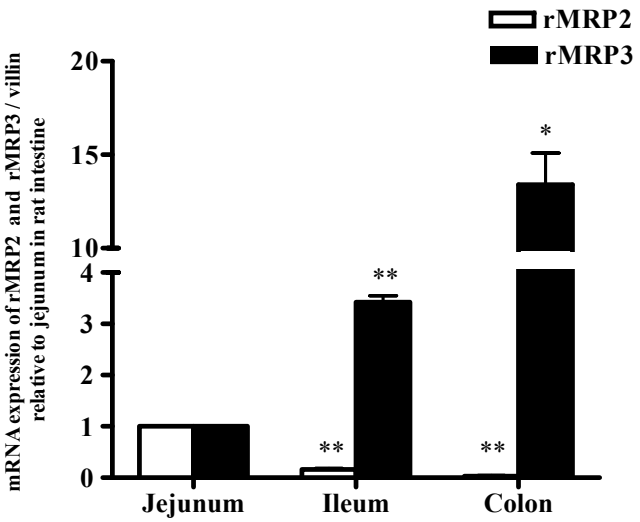

*Figure 4. mRNA expression of rMRP2 and rMRP3 transporters relative to villin expression in intestinal tissue (jejunum, ileum and colon) of the Wistar rat. The mRNA expression of rMRP2 and rMRP3 transporters relative to villin in ileum and colon was expressed relative to that in the jejunum, which was set to unity. Each bar represents the results of three animals ± S.E.M. Significant differences between ileum and colon compared to jejunum are indicated with * $P < 0.05$ and ** $P < 0.001$.*

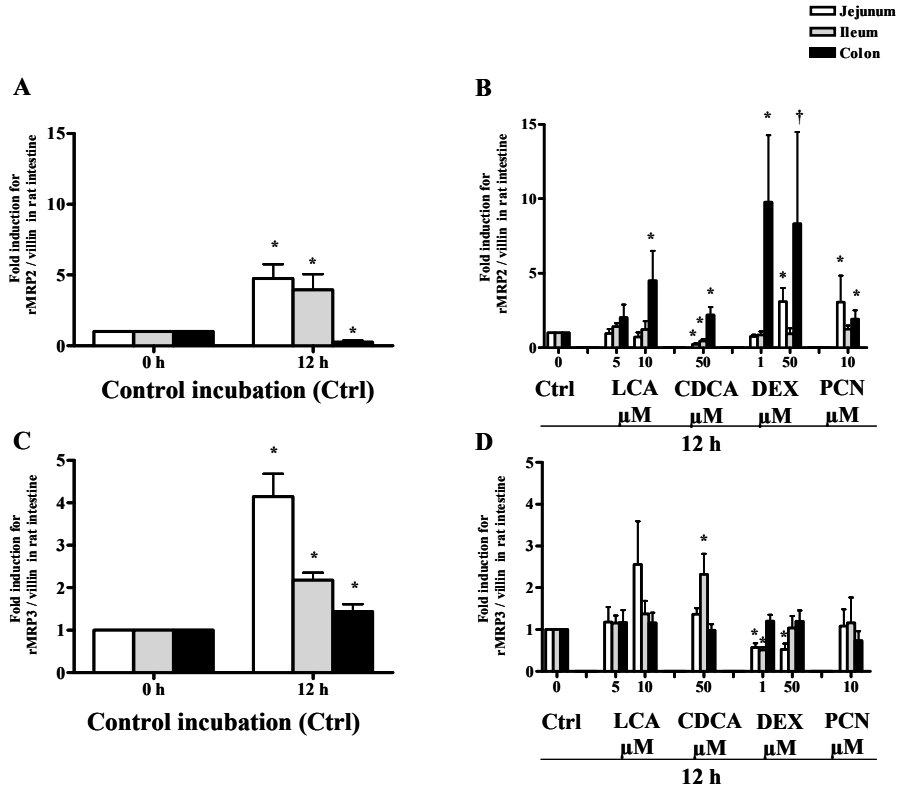

*Figure 5. Rat jejunum, ileum and colon tissue-slices were exposed to LCA (5 µM and10 µM), CDCA (50 µM), DEX (1 µM and 50 µM) and PCN (10 µM) for 12 h after which total RNA was isolated and mRNA expression of rMRP2 (A and B) and rMRP3 (C and D) were evaluated by qRT-PCR. Results were expressed as fold induction after normalizing with villin expression and compared to the control incubated slices of the same segment for 12 h, which was set to 1. Results showed mean ± S.E.M. of 3-5 rats; in each experiment, 6 slices were incubated per condition. Significant differences compared to the control incubations are indicated with * P < 0.05. "†"denotes induction of rMRP2 in all experiments, but failed to reach statistical significance due to large variation between the experiments.*

Expression and regulation of hMRP2 and hMRP3 in human ileum slices. In the human ileum slices, the hMRP2 expression was significantly increased during control incubation for 8 h and returned to control values at 24h, whereas hMRP3 was increased after 24 h (Fig. 6A). LCA induced hMRP3 mRNA expression by 4-fold after 8 h of incubation and hMRP2 expression by 4-fold after 24 h of incubation as compared to the solvent treated controls (Figs. 6B and C). CDCA did not affect the expression of hMRP2 and hMRP3 in human ileum slices during 8 h of incubation but upon prolonged (24 h) exposure of human ileum slices to CDCA, hMRP3 but not hMRP2 expression was induced (Figs. 6B and C). DEX induced hMRP2 expression in all the tested human ileum samples without affecting hMRP3 expression after 24 h incubation (Figs. 6B and C).

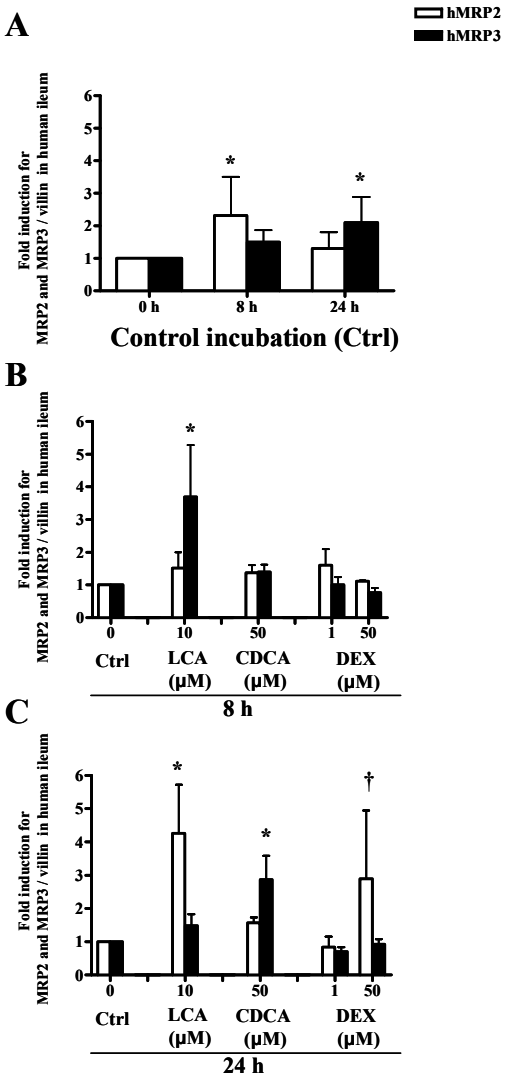

*Figure 6. Human ileum slices were exposed to LCA (10 μM), CDCA (50 μM) and DEX (1 μM and 50 μM) for 8 h and 24 h after which total RNA was isolated and mRNA expression of hMRP2 and hMRP3 (A, B and C) were evaluated by qRT-PCR. Results were expressed as fold induction after normalizing with villin expression and compared to the control incubated slices for 8 h and 24 h, which was set to 1. Results showed mean ± S.E.M. of 4-5 human ileum donors. In each experiment, 3 ileum slices were incubated per condition. Significant differences compared to the control incubations are indicated with * P < 0.05. "†"denotes induction of hMRP2 in all experiments, but failed to reach statistical significance due to large variation between the experiments.*

Expression and regulation of the bile acid synthesis enzyme, transporters and nuclear receptors in rat liver slices. The expression of rCYP7A1 mRNA in rat liver slices was highly sensitive to incubation. rCYP7A1 mRNA expression was decreased by 90% during 8 h of incubation, and upon 24 h incubation, rCYP7A1 mRNA was barely detectable (Fig. 7A). Incubation of rat liver slices with LCA for 8 h significantly decreased the rCYP7A1 expression when compared to control incubated slices (Fig. 7B). Furthermore, LCA induced rSHP and decreased rHNF1α, rLXRα and rLRH-1 expression without affecting the rHNF4α expression after 8 h of incubation (Figs. 8B and C). Prolonged exposure of rat liver slices to LCA for 24 h significantly decreased rHNF4α expression (Fig. 8C). LCA decreased the rPXR and rFXR mRNA expression (Figs. 8B and C). DEX but not PCN

significantly decreased the rCYP7A1 expression with concomitant induction of rSHP (Figs. 7B and 8B). Furthermore, DEX but not PCN induced the rPXR expression in liver slices upon 8 h of incubation (Fig. 8B), whereas, both DEX and PCN significantly decreased the expression of rLXRα, rPXR, rFXR, rHNF1α, rHNF4α and rLRH-1 upon 24 h of incubation (Fig. 8C).

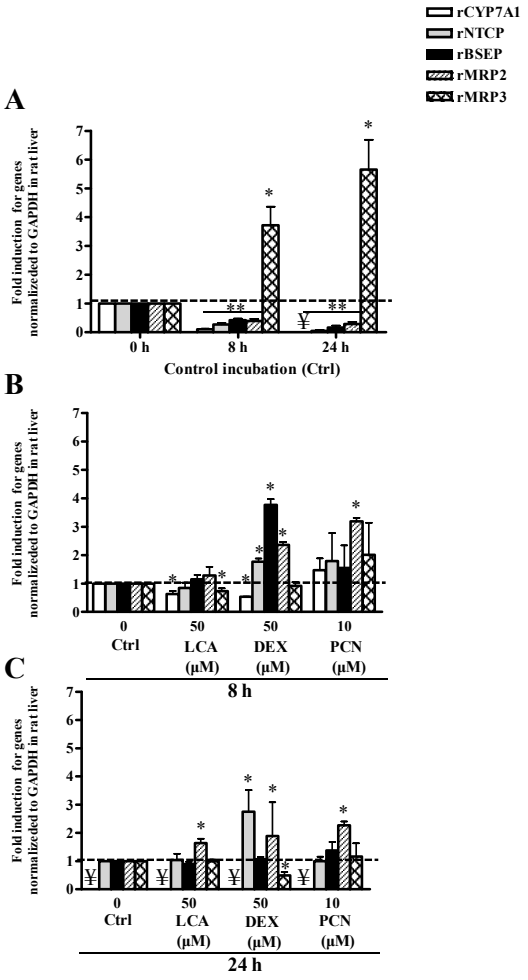

*Figure 7. Slices from rat liver were exposed to LCA (50 μM), DEX (50 μM) and PCN (10 μM) for 8 and 24 h, after which total RNA was isolated and mRNA expression of rCYP7A1, rBSEP, rNTCP, rMRP2 and rMRP3 were evaluated for control (0 h, 8 h and 24 h) (A), (B) 8 h and (C) 24 h incubations by qRT-PCR. Results were expressed as fold-induction after normalizing with rGAPDH and compared with the control slices that were incubated for 8 h and 24 h, which was set to unity. Results showed mean ± S.E.M. of 3-5 rats; 3 slices were incubated per condition in each experiment. Significant differences compared to the control incubations are indicated with * $P < 0.05$ and ** $P < 0.001$. "¥" indicates rCYP7A1 is not detectable in samples incubated for 24 h. Note: The data for rPXR induction in livers slices for 8 h is used from our recent publication (Khan A.A. et.al., 2009).*

In the rat liver slices, the expression of rBSEP and rNTCP mRNA was decreased during control incubation (Fig. 7A). LCA did not affect rBSEP and rNTCP expression (Figs. 7B and C), whereas DEX but not PCN induced rNTCP and rBSEP expression (Fig. 7B and C). Furthermore, the induction of rBSEP by DEX was completely abolished upon 24 h incubation (Fig. 7C). During incubation of rat liver slices, rMRP2 expression was

decreased and rMRP3 expression was increased, (Fig. 7A). LCA decreased the expression of rMRP3 without affecting the expression of rMRP2 in liver slices upon 8 h incubation, but induced rMRP2 expression upon 24 h incubation (Figs. 7B and C). DEX and PCN induced the rMRP2 but not the rMRP3 expression (Figs. 7B and C).

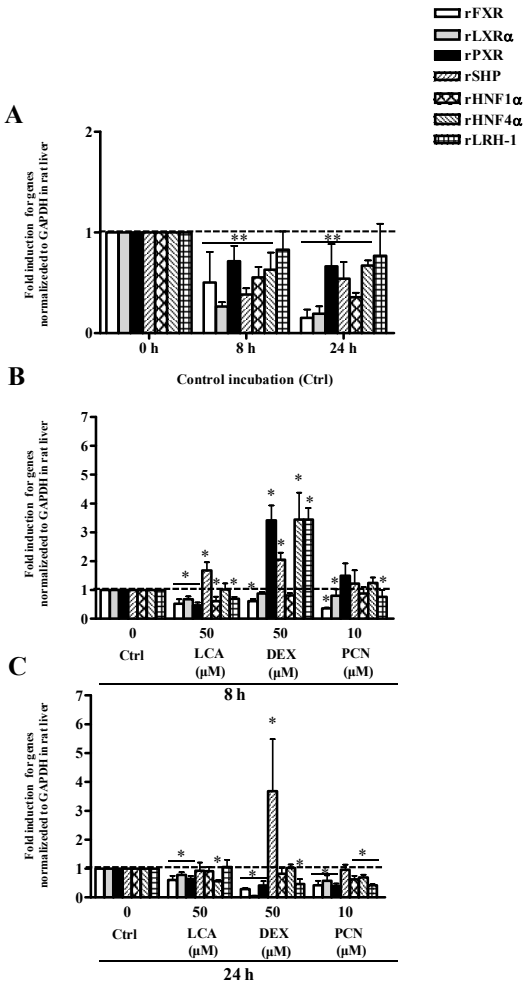

*Figure 8. Slices from rat liver were exposed to LCA (50 μM), DEX (50 μM) and PCN (10 μM) for 8 and 24 h, after which total RNA was isolated and mRNA expression of rFXR, rLXRα, rPXR, rSHP, rHNF1α, rHNF4α, and rLRH-1 was evaluated for control (0 h, 8 h and 24 h) (A), 8 h (B) and 24 h (C) incubations by qRT-PCR. Results are expressed as fold-induction after normalizing with rGAPDH and compared with the control incubated slices for 8 and 24 h, which was set to unity. Results showed mean ± S.E.M. of 3-5 rats; 3 slices were incubated per condition in each experiment. Significant differences compared to the control incubations are indicated with * $P < 0.05$ and ** $P < 0.001$.*

In the rat liver slices, the expression of rBSEP and rNTCP mRNA was decreased during control incubation (Fig. 7A). LCA did not affect rBSEP and rNTCP expression (Figs. 7B and C), whereas DEX but not PCN induced rNTCP and rBSEP expression (Fig. 7B and C). Furthermore, the induction of rBSEP by DEX was completely abolished upon 24 h incubation (Fig. 7C). During incubation of rat liver slices, rMRP2 expression was decreased and rMRP3 expression was increased, (Fig. 7A). LCA decreased the expression of rMRP3 without affecting the expression of rMRP2 in liver slices upon 8 h incubation,

but induced rMRP2 expression upon 24 h incubation (Figs. 7B and C). DEX and PCN induced the rMRP2 but not the rMRP3 expression (Figs. 7B and C).

Expression and regulation of the bile acid synthesis enzyme, transporters and nuclear receptors in human liver slices. In the human liver slices, the expression of most genes including hMRP2 and hMRP3 and CYP7A1 was constant during control incubations for 24 h, only NTCP and FXR expression was decreased and SHP expression was increased (Figs. 9A and B).

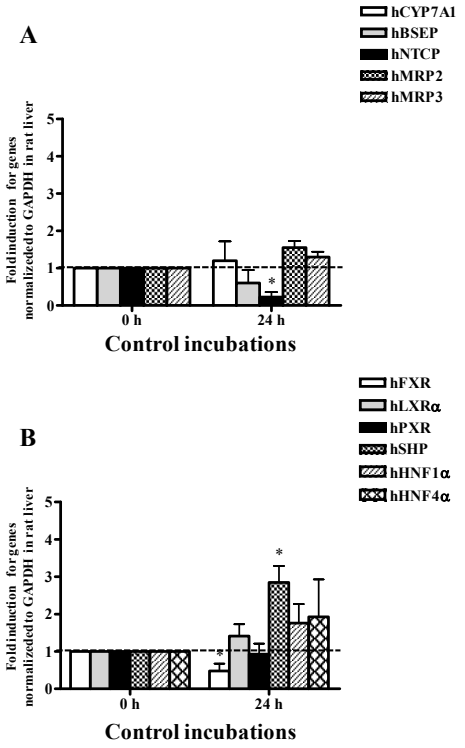

*Figure 9. Slices from human liver were incubated for 24 h, after which total RNA was isolated and mRNA expression of hCYP7A1, hBSEP, hNTCP, hMRP2 and hMRP3 (A) and hFXR, hLXRα, hPXR, hSHP, hHNF1α and hHNF4α, (B) was evaluated by qRT-PCR. Results are expressed as fold-induction after normalizing with hGAPDH and compared with the un incubated samples, which was set to unity. Results show mean ± S.E.M. of 5-7 human liver donors. In each experiment 3 slices were incubated per condition. Significant differences compared to the control incubations are indicated with * P < 0.05.*

The effects of LCA on the expression of bile acid synthesis enzyme and transporters, and the transcription factors regulating their expression in human liver slices were quite variable among the individual livers and therefore the data is given for each individual liver in table 3 as fold induction with respect to the solvent incubated controls. This high variation does not seem to be caused by differences in viability of the livers (as judged by ATP concentration and morphology), nor does it seem to be related to the type of donor (transplantation or partial hepatectomy), or to the expression level of hVDR, hFXR or hPXR. The expression levels of these NR's varied up to 30 fold between the livers for hFXR and up to 16 fold for hPXR (results not shown), whereas hVDR was low but detectable (Ct 33-39) in 4 livers and undetectable (Ct < 40) in 2 livers. In each individual liver, a fold induction of > 1.5 is considered as up regulation and a fold induction of < 0.7 is

considered as down regulation. Furthermore, the expression of a gene is considered as induced or down regulated, if it is induced or repressed in 50% of the human livers, and the others being non responsive.

In contrast to the findings in rat liver slices, incubation of human liver slices with LCA did not consistently up regulate CYP3A4 expression (up regulation in four, down regulation in three livers) (table 3), whereas DEX showed a strong up regulation of CYP3A4 expression in all the human livers (table 4). In addition LCA did not have a consistent effect on hCYP7A1 (decrease in three out of seven livers), hSHP expression, (induction in only two out of seven livers), hHNF1α expression (reduced in three out of five livers), hLXRα (decreased in three out of five livers) and hHNF4α (decreased in four out of six livers) (table 3). DEX induced hHNF4α expression in three out of four livers, but hLXRα expression was not affected by DEX (table 4).

The effect of LCA on the expression of hBSEP, hNTCP, hPXR, hFXR, hMRP2 and hMRP3 is neither significant nor consistent (table 3). DEX significantly induced hBSEP and moderately induced hPXR expression in all the livers, and induced hNTCP in four out of five livers, but had no effect on hMRP2 and hMRP3 expression (table 4).

Discussion

In this report, rat and human precision-cut intestinal and liver slices were used to characterize the role of LCA in the regulation of genes involved in bile acid detoxification, transport and synthesis. Our data on the effects of $1,25(OH)_2D_3$ (33, 34), BUD, GW4064, CDCA, PCN and DEX show that VDR, FXR, PXR and GR pathways are intact in the tissue slices. The observed changes in the expression of the CYP P450 isozymes and transporters during control incubations indicate that apparently the basal expression is normally maintained by ligands that are absent in the culture medium. The pattern of LCA-mediated induction of CYP3A1 and CYP3A2, and CYP3A4 in the rat and the human intestine, respectively (Figs. 1A, 1B, 1C and 3B) resembles that of the VDR ligand, $1,25(OH)_2D_3$, and was clearly different from that of FXR, PXR or GR ligands as reported earlier by us (33) and others (20), confirming the role of VDR in the regulation of CYP3A isozymes by LCA in the rat and human intestine. However, the higher induction of CYP3A1 mRNA by LCA in the rat ileum compared to the colon cannot be explained by differences in VDR expression, as VDR was shown to be higher in colon (33), thus other factors such as the presence of activators/repressors may also play a role. The induction of CYP3A9 by LCA in the rat intestine is likely mediated via PXR, as the enzyme was induced by other PXR ligands, PCN and DEX, but not by $1,25(OH)_2D_3$, the VDR ligand, or the CDCA, FXR ligand (33). The high induction of CYP3A9 in the colon is consistent with the higher expression of PXR (33). In human ileum slices, LCA induced CYP3A4 expression (Fig. 3B). However, the nuclear receptor (NR) involved in the induction of CYP3A4 by LCA is not conclusive from our studies since CYP3A4 was induced by PXR,

GR and VDR ligands (20, 33). The involvement of VDR rather than PXR is suggested recently by Matsubara et al. (44), who showed that the regulation of human CYP3A4 by LCA in HepG2 cells is specifically mediated by VDR and not by PXR.

In the rat liver slices, LCA did not affect CYP3A1 and CYP3A2 mRNA expression (Fig. 2), which is congruent with the results obtained previously with the VDR ligand, $1,25(OH)_2D_3$ (33). The absence of a VDR mediated induction of CYP3A isozymes can be explained by the localization of VDR in the bile duct epithelial cells but not in the hepatocytes as reported earlier by Gascon-Barre et.al. (42), and confirmed in our studies (chapter 4), whereas most of the CYP3A expression is present in the hepatocytes. However, the LCA-induced CYP3A9 expression upon 24 h incubation was similar to that from the PXR ligands, PCN and DEX (Fig. 2), suggesting a PXR response. This delayed response in the induction of CYP3A9 by LCA suggest that this effect might not be directly mediated by LCA, but by its metabolite, 3KCA, as it was shown that LCA itself is a poor PXR ligand, and needs to be metabolized to 3KCA prior to PXR binding (41, 53).

In contrast to the observations in rat liver, but consistent with those in human ileum, LCA induced the CYP3A4 expression in human liver slices of four out of seven liver donors (Fig. 3). This induction could, in principle, be explained by the observed expression of VDR and its involvement with LCA in human hepatocytes (21). But the induction of CYP3A4 did not correlate with the VDR expression in these livers (table 3). Therefore it is unlikely that the LCA mediated induction of CYP3A4 in these four livers is mediated by VDR; other nuclear receptors such as PXR with its ligands DEX (table 4) and rifampicin (47), could explain this induction data. This variability in effect may be caused by the inter individual variation in activity of the enzymes that are involved in the metabolism of LCA, sulfotransferases (27) and CYP3A4 (36), which may result in variations in the effective exposure to LCA. Subsequent to characterizing the effect of LCA on CYP3A isozymes in rat and human intestine and liver, we studied the effect of LCA on the regulation of rMRP2 and rMRP3 in intestinal slices, since these transporters play an important role in the excretion of monovalent and conjugated bile acids across the apical and basolateral membranes, respectively, of enterocytes (8, 10, 25, 26, 61) and the basolateral transporter, OSTα-OSTβ (5, 13). Recently, we showed that LCA decreased the expression of OSTα and OSTβ in the rat ileum and induced OSTα and OSTβ in the rat colon and liver, and in the human ileum and liver (Khan et. al., 2009b). The reciprocal expression of rMRP3 and rMRP2 along the length of the rat intestine with rMRP3 expression decreasing from jejunum and rMRP2 expression increasing towards the colon (Fig. 4), together with the basolateral localization of OSTα-OSTβ in the ileum (4), suggest that BA transport in the colon is directed more towards the lumen, whereas in the small intestine basolateral transport is predominant. The finding that LCA did not affect the rMRP3 expression in rat jejunum, ileum and colon slices but induced rMRP2 expression in colon slices (Figs. 5B and D) and decreased rOSTα-rOSTβ in rat ileum (34) suggests that the luminal excretion of BA's is further stimulated by LCA. Although the VDR ligand,

$1,25(OH)_2D_3$ also induced rMRP2 without affecting rMRP3 expression (A.A. Khan et. al., unpublished observation), we cannot conclude whether the effect of LCA on rMRP2 is mediated by VDR, PXR or FXR, as DEX, PCN and CDCA also induced rMRP2 expression in the rat colon (Fig. 5B). In the mouse colon, however, Mrp3 was reported to be induced by $1,25(OH)_2D_3$ (45), suggesting species differences in the regulation of MRP3. Thus, in the rat, LCA favours its own detoxification and transport into the lumen of the colon by inducing rMRP2 but not rMRP3 expression (Figs. 5B and D), hence excretion via the faeces. In contrast, the primary BA, CDCA, stimulates absorption of bile salts by induction of rMRP3 and OSTα-OSTβ expression and repression of rMRP2 expression in rat jejunum and ileum (Figs. 5B and D), favouring the reclamation of bile acids in the small intestine. Also in the human intestine, LCA may induce the luminal transport of BAs by inducing hMRP2 expression, whereas CDCA favours the basolateral transport of BAs by inducing hMRP3 expression in ileum slices (Figs. 6B and C), which is consistent with the earlier reports (29). As CDCA did not affect hMRP2 expression in human ileum slices, the LCA effects are not likely to be mediated by FXR.

In addition to the evaluation of the effects of LCA on the detoxification enzymes and MRP transporters in rat and human intestine, we studied the effect of LCA in rat and human liver on CYP7A1, the rate limiting enzyme in bile acid synthesis, and on the bile acid transporters, NTCP, BSEP, MRP2 and MRP3 and the NR/transcription factors involved in the regulation of these proteins. In rat liver slices, LCA decreased the rCYP7A1 expression, with simultaneous induction of rSHP, as expected for an FXR ligand, and in line with the effects of CDCA (chapter 4). Furthermore, in rat liver slices, LCA also affected the SHP-independent pathways of rCYP7A1 regulation by decreasing the expression of rHNF1α, rHNF4α, rLXRα and rLRH-1 (Figs. 8B and C), that are essential for the expression of rCYP7A1 (1, 14, 15, 22, 38). However, the effects of LCA are not likely to be mediated by PXR since by PCN, the PXR ligand, failed to alter the expression of rCYP7A1 and rSHP. The effects of LCA on rCYP7A1 seem to decrease with increasing incubation time, which might be due to the metabolism of LCA into metabolites which are less efficient agonists of FXR but have a higher affinity towards PXR. Therefore, the FXR effects of LCA, such as repression of bile acid synthesis may precede the PXR effects, like CYP3A9 induction. Furthermore, we found that LCA, like CDCA decreased the expression of rFXR and rPXR in rat liver slices (Fig.8B) (chapter 4), an observation not reported previously for the rat liver. Thus, in addition to decreasing the expression of rCYP7A1, LCA also decreased the rFXR expression, probably by a feedback loop mediated by the decreased expression of rHNF1α, (Figs. 8B and C), which is required for its basal expression (39). The LCA dependent rSHP induction and concomitant repression of rPXR is consistent with results found in human hepatocytes and in mice with SHP-mediated repression of PXR (48), but in contrast with the increased expression of SHP and PXR found in mice fed with CDCA and GW4064 (31).

Table 3 Summary of the effects of LCA on the expression genes in human livers; n= 4-7 human liver donors; criteria for induction and repression are 1.5-fold and 0.7-fold, respectively.

Human livers			HL1	HL2	HL3	HL4	HL5	HL6	HL7	Mean	S.E.M.	*p*
Gender			Female	N/A	Male	N/A	Female	Female	Female			
Age			54	N/A	65	N/A	72	64	42			
ATP pmol / µg of protein ± SD			*10.4 ± 1.5	*5.7 ± 1.9	*12.1 ± 1.0	11.1 ± 0.9	*3.3 ± 1.2	*9.7 ± 1.8	ND			
VDR (ΔC_T)			11.7	16.2	14.8	16.2	NDE	NDE	ND			
Gene												
LCA	↔	CYP3A4	0.6	0.6	1.8	0.5	2.2	3.2	2.0	1.54	0.38	0.175
	↔	CYP7A1	↔	↔	0.3	0.5	↔	0.1	1.9	0.90	0.25	0.683
	↔	SHP	↔	0.7	0.7	↔	2.6	3.1	↔	1.40	0.38	0.312
	□	HNF1α	↔	↔	0.3	↔	↔	ND	↔	0.78	0.10	†0.042
	↔	HNF4α	↔	0.4	0.2	0.4	2.4	ND	0.4	0.80	0.34	0.523
	↔	LXRα	0.6	0.7	0.4	ND	↔	↔	ND	0.77	0.19	0.166
	↔	PXR	0.7	0.3	1.7	ND	1.5	0.4	ND	0.93	0.30	0.794
	↔	FXR	0.6	↔	0.5	ND	↔	↔	ND	0.86	0.16	0.336
	↔	BSEP	0.4	↔	1.7	↔	0.7	0.2	↔	0.84	0.19	0.413
	↔	NTCP	0.3	0.30	↔	0.1	2.3	0.1	2.2	0.86	0.36	0.712
	↔	MRP2	0.5	↔	0.5	↔	↔	↔	1.5	1.03	0.16	0.867

HL-human liver; ND-not done; NDE-not detectable; N/A – not available; ↔ - No significant effect; ↓- significant repression; "" data is taken from Khan et al.(33); All values are expressed as fold induction with respect to their solvent incubated controls. † indicates all samples showed down regulation but to a different extent, which is outside the criteria for induction and repression.*

Table 4 Summary of the effects of DEX on the expression genes in human livers; n= 4-5 human liver donors; criteria for induction and repression are 1.5-fold and 0.7-fold, respectively.

Human livers			HL1	HL2	HL3	HL4	HL5	Mean	S.E.M.	*P*
Gender			Female	N/A	Male	Female	Female			
Age			54	N/A	65	72	64			
ATP pmol / μg of protein ± SD			*10.4 ± 1.5	*5.7 ± 1.9	*12.1 ± 1.0	*3.3 ± 1.2	*9.7 ± 1.8			
VDR (ΔC_T)			11.7	16.19	14.8	NDE	NDE			
Gene										
DEX	↑	CYP3A4	17.6	4.0	15.0	1.7	9.1	9.52	3.05	0.023
	↑	HNF1α	↔	↔	1.6	1.7	ND	1.35	0.20	0.079
	↑	HNF4α	1.9	↔	6.5	3.9	ND	3.31	1.24	0.071
	↑	PXR	↔	↔	2.3	↔	1.6	1.5	0.21	0.043
	↑	BSEP	8.7	2.3	15.2	3.3	3.6	6.61	2.40	0.048
	↑	NTCP	6.5	1.5	4.2	2.3	↔	3.04	1.03	0.083
	↔	MRP2	↔	1.9	0.6	↔	↔	1.2	0.22	0.387
	↔	MRP3	0.5	↔	2.0	ND	0.5	0.95	0.35	0.861

HL-human liver; ND-not done; NDE-not detectable; N/A – not available; ↔ - No effect; ↑- significant induction or induction in ≥50% of the livers; "" data is taken from Khan et al.(33); All values are expressed as fold induction with respect to their solvent incubated controls.*

In human liver slices, the effects of LCA on the NRs were highly variable, as observed for CYP3A4. The decreased HNF1α, HNF4α and LXRα, as found in rat liver slices, was observed in only 3-5 of the tested human livers. (table 3 and Figs. 8B and C). Unlike in rat liver slices, LCA did not affect the hMRP2 expression in human livers (Figs. 7B and C, table 3). In addition, the LCA mediated effects on the expression of hSHP, hBSEP and hNTCP in human livers were not consistent, but CDCA effects were consistent with an intact FXR pathway (chapter 4). Hence, it is concluded that LCA does not act as an FXR ligand in the human liver, as also reported by others (2, 30). These results indicate that the cholestatic effects of LCA are at least partly mediated by direct effect of LCA itself on the regulation of hBSEP but not on hMRP2. Furthermore, DEX induced hNTCP (table 4), similar to that of rNTCP (Figs. 8B and C), which is probably mediated by GR and consistent with an earlier report (18). Hence, the GR pathway is intact in human liver slices. DEX also induced hBSEP, similar to rBSEP (table 4 and Fig. 8B), which is not reported before.

In conclusion, LCA plays an important role in the feed forward regulation of its detoxification pathways in the rat and human intestine by inducing CYP3A isozymes, thereby increasing its metabolism. In addition, it increases the luminal efflux of conjugated (toxic) bile acids via rMRP2 into the colon, while simultaneously preserving the primary bile acid pool by inducing the expression of rOSTβ in the colon and rMRP3 in the ileum. Distinct species differences were observed for the effects of LCA in the rat and human liver. In the rat liver, LCA decreases bile acid synthesis and excretion but its effects in the human liver were inconsistent and need further investigation. Thus, LCA as a promiscuous ligand for FXR, VDR and PXR, regulates bile acid synthesis, metabolism and transport in the rat intestine and liver and the human intestine. This altered expression of transporters and CYP3A enzymes may also have consequences for the disposition of drugs, especially in situations when LCA is increased such as during cholestasis.

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Chapter 4

1α,25-dihydroxyvitamin D_3 mediates down regulation of HNF4α, CYP7A1 and NTCP in human but not in rat liver

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Abstract:

We studied the regulation of expression of proteins involved in bile acid synthesis and transport by the vitamin D receptor (VDR) and compared it to the effects of farnesoid X receptor (FXR) in rat and human livers. Rat and human liver slices were treated with the VDR ligand, 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and the FXR ligand, chenodeoxycholic acid (CDCA), and the mRNA expression of the bile acid synthesis enzyme, CYP7A1, the bile acid transporters, BSEP, NTCP, MRP2 and MRP3, and nuclear receptors/transcription factors, VDR, FXR, LXRα, PXR, SHP, HNF1α, HNF4α, LRH-1 were assessed. $1,25(OH)_2D_3$ did not affect the expression of bile acid syntheses enzymes, transporters and nuclear receptors/transcription factor, except for rFXR and rPXR, which were decreased in rat liver slices. In human liver slices, $1,25(OH)_2D_3$ significantly decreased CYP7A1 and NTCP expression, with simultaneous repression of HNF4α. CDCA also decreased CYP7A1 and NTCP but increased SHP and BSEP expression in human and rat liver slices. In addition, LRH-1 and HNF4α expression was also decreased in rat liver slices. In conclusion, VDR is involved in bile acid homeostasis in humans but not rat liver by decreasing the expression of CYP7A1 and the NTCP, FXR target genes. The appreciable species specific difference in the regulation of CYP7A1 and NTCP by the VDR ligand in rat and human liver can be attributed to the localization of VDR merely exclusively in the non-parenchymal cells in the rat liver whereas in the human livers, VDR was located in the parenchymal cells as well as in the non-parenchymal cells.

Keywords: CYP7A1, regulation, nuclear receptors, intestinal slices, liver slices

Introduction

Bile acids (BAs) are synthesized from cholesterol (37) in the liver by a cascade of 12 reactions, initiated by the first and rate limiting step, cholesterol 7α-hydroxylation, catalyzed by cytochrome P450 7A1 or CYP7A1 (6) to form chenodeoxycholic acid (CDCA) and cholic acid (CA), which upon amidation**,** are secreted into the bile canaliculi by the bile salt export pump (BSEP) and transported into the intestine from the bile. About 95 % of the bile acids are actively re-absorbed in the terminal ileum by the coordinate action of apical sodium dependent bile acid transporter (ASBT) and the basolateral heterodimeric organic solute transporter (OSTα-OSTβ) (3, 38). In the liver, bile acids are taken up into hepatocytes by the sodium dependent co transporting polypeptide (NTCP) and members of the organic anion transporting polypeptide (OATP) family. Bile acid synthesis and secretion in the liver, reabsorption in the ileum and uptake in the liver constitute the enterohepatic cycle (19), which maintains the bile acid pool in vivo and is co-ordinately modulated by regulation of CYP7A1, BSEP and NTCP in the liver, and ASBT and OSTα-OSTβ in the ileum.

Bile acid synthesis and disposition in the liver and ileum are highly regulated, and the involvement of bile acid activated nuclear receptors, farnesoid X receptor (FXR, NR1H4) and pregnane X receptor (PXR, NR1I2) was demonstrated (35). The ligand-activated FXR heterodimerizes with the retinoic acid X receptor α (RXRα, NR2B1) and inhibits CYP7A1 and NTCP expression in the liver (10, 15) and ASBT expression in the ileum (30) by inducing short heterodimer partner (SHP, NR0B2), which inhibits liver receptor homologue-1 (LRH-1) that is required for their basal expression (4, 15). BSEP is positively regulated by ligand-bound FXR by binding to the inverted repeat (IR1) in the promoter region (2). CYP7A1 is also negatively regulated by bile acids through a SHP-independent pathway by activating c-Jun kinase by protein kinase C, which inactivates the hepatic nuclear factor 4α (HNF4α) by phosphorylation (16), a transcription factor that is essential for CYP7A1 expression (6, 36). Furthermore, the hormones, fibroblast growth factor 15 (FGF15) and FGF19, synthesized in the rat and human intestine, respectively, upon bile acid-mediated activation of FXR, acts as an enterohepatic signaling protein for feedback inhibition of CYP7A1 expression upon binding to the fibroblast growth factor receptor 4 (FGFR4) of the hepatocyte (20). The tumor necrosis factor β (TGFβ) and cytokines also regulate CYP7A1 expression (8, 27). CYP7A1 expression is also subjected to posttranslational regulation (31). Furthermore, CYP7A1 is positively regulated by LXRα, which upon ligand binding heterodimerizes with the RXRα and binds to its response elements (LXRE) in the CYP7A1 promoter and induces its expression in the rodents but not in man because a DR4 motif is absent in the CYP7A1 promoter (26).

Although it has been shown that bile acids such as lithocholic acid (LCA), and to a lesser extent, CDCA, bind to the vitamin D receptor (VDR, NR1I1) (29), the involvement of the VDR in the regulation of bile acid synthesis and transport proteins in the liver has not

been extensively studied to date. Recently, we studied the VDR mediated regulation of the OSTα and OSTβ by treating rat and human intestine and liver precision-cut slices with $1,25(OH)_2D_3$, the natural ligand of VDR, and found a clear organ- and species-specific differences in rat and man (24). In addition, Chen et al. (5) showed that intraperitoneal administration of $1,25(OH)_2D_3$ to Sprague-Dawley rats for four days increased bile acid concentrations in the portal blood subsequent to the induction of ASBT protein in the ileum. Further, these rats exhibited altered expression of the enzymes for bile acid synthesis (CYP7A1) and transporters in small intestine, colon, and liver (7). These in vivo effects might be mediated by $1,25(OH)_2D_3$ directly, as shown by Han et al. (17) in human hepatocytes and HepG2 cells, or could be due to FGF15/FGF19 as well as indirect FXR-related effects that are associated with the increased bile acid concentrations in the portal circulation (7).

In this communication, we studied the VDR-mediated regulation induced by $1,25(OH)_2D_3$ on genes that are involved in bile acid synthesis and transport, and genes that encode the nuclear receptors/transcriptions factors that are involved in the regulation of bile acid disposition in human and rat liver precision-cut liver slices. Liver slices represent a valuable ex-vivo model to study the regulation of genes of interest under identical, controlled and nearly physiological conditions in different species (32). In addition we compared the localization of the VDR protein in the human and rat liver by immunohistochemistry.

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 hepactectomy for the removal of carcinoma or from redundant parts of donor livers remaining after split liver transplantation, as described previously by Olinga et al. (33). The human liver donor characteristics were as reported earlier (25), and two additional human livers were used to study the effects of $1,25(OH)_2D_3$ and the liver donor characteristics are reported in (A.A. Khan et al., manuscript submitted). 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 (UMCG), Groningen, respectively.

$1,25(OH)_2D_3$ in ethanol was purchased from BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA. Chenodeoxycholic acid (CDCA) was purchased from Calbiochem, San Diego, CA, USA. 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). D-Glucose and HEPES were procured from ICN Biomedicals, Inc. (Eschwege, Germany). Low gelling temperature agarose and 3-amino-9-ethylcarbazole (ACE) were

purchased from Sigma–Aldrich (St. Louis, MO). RNAeasy mini columns were obtained from Qiagen, Hilden, Germany. Rabbit anti-rat Cytokeratin (CK)-7 and rabbit anti-rat vitamin D receptor (VDR) antibody was purchased from Santa Cruz Biotechnology, Inc., Heidelberg, Germany. Rabbit anti-human VDR antibody was purchased from Fitzgerald Industries International, Inc., Concord, USA. Goat Anti-Rabbit Immunoglobulin / Biotinylated (GARBio) and the Avidin-Biotin (AB) complex was procured from Dakocytomation, Glostrup, Denmark. 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 liver slices. Rat and human liver slices (8 mm diameter, 200 to 300 µm thick, wet weight of 10–12 mg) were prepared according to the earlier published method (25, 33). In brief, cylindrical cores of 8 mm were prepared from rat and human liver tissue by advancing a sharp rotating metal tube in the liver tissue and were subsequently placed in the pre-cooled Krumdieck tissue slicer (Alabama R&D, Munford, AL, USA) containing carbogenated ice-cold KHB and slicing was performed with the standard settings (cycle speed 40: interrupted mode). Subsequently, slices were stored in ice-cold UW solution on ice prior to the start of the experiment, which usually varies from 1 to 3 h after sacrificing the rat and for human livers 2 to 3 h post surgery.

Immunohistochemical localization of VDR in rat and human liver. Rat and human liver tissue were embedded in Tissue–Tek, and frozen in isopentane, kept at -80° C and stored at -80° C, until cryosectioning. Tissue sections of 4 µm thick were cut by a cryostat (Lieca CM 3050) at -20° C, placed on Super-Frost plus slides (Menzel, Braunchweig, Germany) and air-dried. Sections were fixed in acetone for 10 min at room temperature and then allowed to rehydrate in PBS for 5 min.

For VDR staining, rat and human liver sections were incubated with the respective rabbit VDR primary antibodies at several dilutions in PBS that was supplemented with 1% rat or human serum for 1 h. Sections were rinsed and washed with PBS to remove the excess of primary antibody and then incubated with H_2O_2 (62.5 µl of 30% H_2O_2 in 50 ml of PBS), avidin/biotin sequentially for 1 h, each with intermittent washings with PBS to block endogenous peroxidase and avidin/biotin. After the rinse of the sections with PBS, sections were incubated with secondary antibody, Goat anti-rabbit labelled with biotin (GARBio) and Avidin/Biotin complex labelled with horseradish peroxidase, sequentially for 30 min each with intermittent washings. The sections were later stained with 3-amino-9-ethylcarbazole (AEC) and haematoxylin.

For rat cytokeratin (CK)-7 staining, sections were placed on Super-Frost plus slides and air-dried, then fixed in acetone for 10 min at room temperature and allowed to rehydrate in PBS for 5 min. Rat liver sections were incubated with the respective rabbit anti-rat CK-7 primary antibody at 1:50 dilution in PBS, which was supplemented with 1% rat serum for 1 h. Sections were rinsed and washed with PBS to remove the excess of primary antibody and incubated with H_2O_2 (62.5 µl of 30% H_2O_2 in 50 ml of PBS) for 1 h to block any endogenous peroxidase. After the rinse with PBS, sections were incubated with a secondary antibody, goat anti-rabbit IgG labelled with horseradish peroxidise (GARPO) for 30 min, then stained with AEC for 20 min and with hematoxylin.

Induction studies. 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 William's 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 liver slices were incubated with $1,25(OH)_2D_3$ (final concentrations, 10-200 nM) and CDCA (final concentrations, 10-100 µM). Human liver slices were incubated with $1,25(OH)_2D_3$ (final concentrations, 100 and 200 nM) and CDCA (final concentration, 100 µM). $1,25(OH)_2D_3$ and CDCA, as a 100-fold concentrated stock solution in ethanol and methanol, respectively, were added in appropriate aliquots. Control liver slices were incubated in supplemented Williams medium E with 1% ethanol or 1% methanol without the inducers. Rat and human liver slices were incubated for 8 h and 24 h, respectively, since rat rCYP7A1 mRNA expression was found to be sensitive to incubation conditions and were barely detectable after 24 h incubation, whereas for human liver slices, CYP7A1 expression remained stable until 24 h. From each 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 quantitative real-time PCR (qRT-PCR) analysis. Samples were stored in –80° C freezer until RNA isolation. The experiments were repeated in 3 to 5 rat livers and among 4 to 7 human livers.

RNA isolation and qRT-PCR. Total RNA from rat and human liver slices was isolated with RNAeasy mini columns from Qiagen according to the manufacturer's instruction. The total RNA concentration and quality were determined by measuring the absorbance at 260, 230 and 280 nm using a Nanodrop ND100 spectrophotometer (Wilmington, DE USA). The ratio of absorbance measured at 260 over 280 and 230 over 260 was above 1.8 for all the samples, indicating high quality RNA. The total RNA was reverse-transcribed into template cDNA with Promega reverse-transcription kit from Promega Corporation, Madison WI, USA according to the earlier published method (25). qRT-PCR for the rat and human genes of interest was performed using primer sequences given in table 1 and 2 by SYBR green detection system, and hGAPDH was analyzed by Taqman detection system according to the availability of primer sets. PCR conditions were similar to those described

in an earlier report (25). All samples were analyzed in duplicates in 384 well plates using ABI7900HT from Applied Biosystems. 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, GAPDH (ΔC_T), which was compared to the corresponding ΔC_T of the solvent control ($\Delta\Delta C_T$). Data were expressed as fold induction or repression of the gene of interest according to the formula $2^{-(\Delta\Delta CT)}$.

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.

Table 1 Oligonucleotides for quantitative real-time PCR, rat genes (SYBR green)

Gene	Forward primer (5' – 3')	Reverse primer (5' – 3')
rGAPDH	CGCTGGTGCTGAGTATGTCG	CTGTGGTCATGAGCCCTTCC
rCYP7A1	CTGTCATACCACAAAGTCTTATGTCA	ATGCTTCTGTGTCCAAATGCC
rBSEP	TGGAAAGGAATGGTGATGGG	CAGAAGGCCAGTGCATAACAGA
rNTCP	CTCCTCTACATGATTTTCCAGCTTG	CGTCGACGTTCGTTCCTTTTCTTG
rMRP2	CTGGTGTGGATTCCCTTGG	CAAAACCAGGAGCCATGTGC
rMRP3	ACACCGAGCCAGCCATATAC	TCAGCTTCACATTGCCTGTC
rSHP	CTATTCTGTATGCACTTCTGAGCCC	GGCAGTGGCTGTGAGATGC
rHNF1α	CTCCTCGGTACTGCAAGAAACC	TTGTCACCCCAGCTTAAGACTCT
rHNF4α	CCAGCCTACACCACCCTGGAGTT	TTCCTCACGCTCCTCCTGAA
rLXRα	TGCAGGACCAGCTCCAAGTA	GAATGGACGCTGCTCAAGTC
rPXR	GATGATCATGTCTGATGCCGCTG	GAGGTTGGTAGTTCCAGATGCTG
rLRH-1	GCTGCCCTGCTGGACTACAC	TGTAGGGCACATCCCCATTC

Table 2 Oligonucleotides for quantitative real-time PCR, human genes (SYBR Green and Taqman analysis)

Gene	Forward primer (5' – 3')	Reverse primer (5' – 3')
hGAPDH	ACCCAGAAGACTGTGGATGG	TCTAGACGGCAGGTCAGGTC
hCYP7A1	GCTGTTGTCTATGGCTTATTCTT	GCCCAGGTATGGAATTAATCCA
hBSEP	CAGTTCCCTCAACCAGAACAT	TTTGATCATTTCGCTCTCGATG
hNTCP	CTCAAATCCAAACGGCCACAAT	CACACTGCACAAGAGAATGATGATC
hMRP2	CGGACAGCATCATGGCTTCT	ACTCCTTCCTTGGCCAAGTTG
hMRP3	GTCCGCAGAATGGACTTGAT	TCACCACTTGGGGATCATTT
hSHP	TGAAAGGGACCATCCTCTTCA	CAATGTGGGAGGCGGCT
hPXR	CCCAGCCTGCTCATAGGTTC	GGGTGTGCTGAGCATTGATG
hLXRα	CCCTTCAGAACCCACAGAGATC	GCTCGTTCCCCAGCATTTT
hHNF1α	CAGAAAGCCGTGGTGGAGAC	GACTTGACCATCTTCGCCACA
hHNF4α	CCTGGAATTTGAGAATGTGCAG	AGGTTGGTGCCTTCTGATGG
hGAPDH	Assay-on-Demand™ ID Hs99999905_m1	
	Probe sequence - (5'FAM -3' NFQ) GCGCCTGGTCACCAGGGCTGCTTTT	

Results

Immunohistochemical localization of Vitamin D Receptor (VDR) in Rat and Human Liver. In rat liver tissue, VDR was localized in the bile duct epithelial cells (BEC) (Figs. 1C and F), which were identified by the cytokeratin (CK)-7 staining (Figs. 1B and E), a

marker for BEC. Furthermore, VDR staining was observed along the endothelial cells of the portal venules in the rat liver sections. However, rat hepatocytes were devoid of VDR staining. In human liver tissue, VDR staining was observed in the BEC (Figs. 2D and E) as well as in the hepatocytes but not in the endothelial lining of the portal venules (Fig. 2F). Control rat (Figs. 1A and D) and human (Figs. 2A, B and C) liver sections without primary VDR antibody incubation did not show any staining.

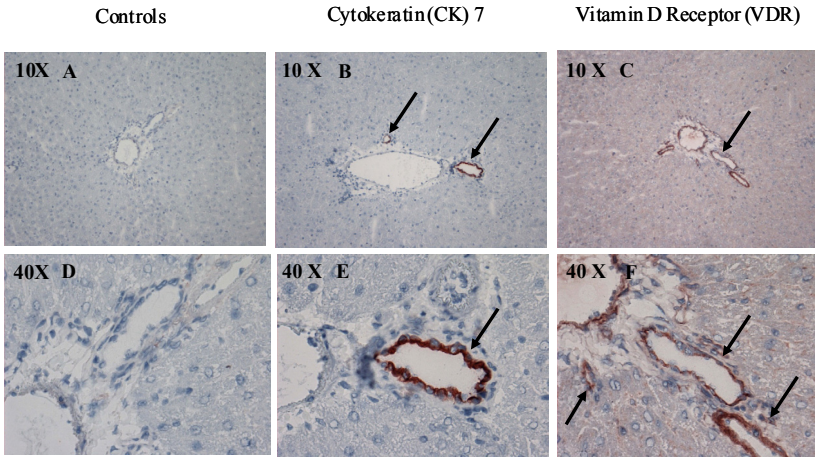

Figure 1. Immunohistochemistry of Wistar rat liver showed that VDR protein is expressed in the bile duct epithelial cells (BEC) (C and F) and a low expression was observed in endothelial cells. Furthermore, BEC was visualized in the liver sections by cytokeratin – (CK)7 staining (B and E). Control liver sections incubated without VDR and CK-7 primary andtibodies did not show any staining (A and D).

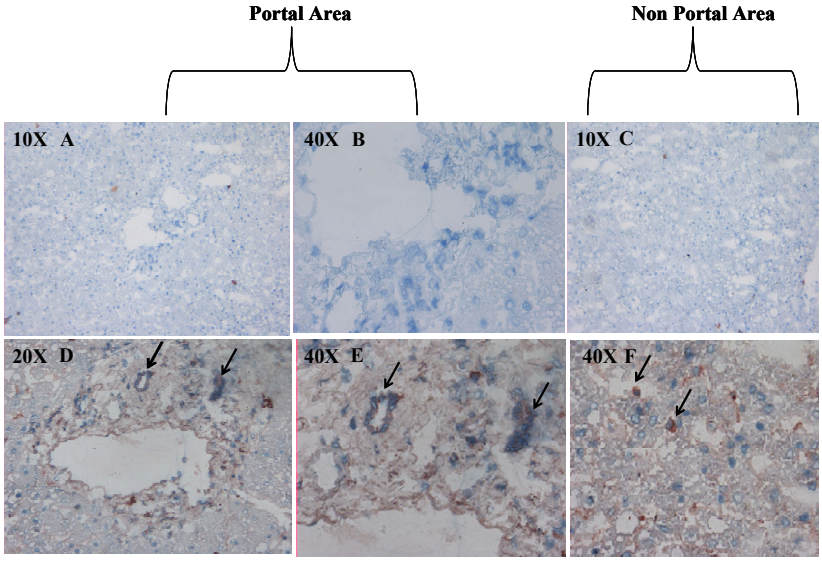

Figure 2. Immunohistochemical staining of human liver showed that VDR protein is expressed more abundantly in BEC (D and E) whereas a lower but detectable expression was observed in hepatocytes (F). Control liver sections which were incubated without the VDR primary antibody did not show any staining (A, B and C).

Effect of VDR and FXR ligands on the expression of the bile acid synthesis enzyme, transporters and nuclear receptors/transcription factors in rat liver slices. The expression of rat rCYP7A1 was highly unstable during the incubation of rat liver slices, and levels decreased after 8 h of incubation (Fig. 3A) and became undetectable at 24 h of incubation. Therefore, the effects of VDR and FXR ligands were studied at 8 h. Pilot experiments showed that the effects of 1,25(OH)$_2$D$_3$ and CDCA on the regulation of the bile acid transporters, NTCP and BSEP, and the nuclear receptors, FXR and HNF4α at 24 h were not different from those obtained at 8 h (data not shown). Incubation of rat liver slices for 8 h with the VDR ligand, 1,25(OH)$_2$D$_3$ did not affect the expression of rCYP7A1, whereas CDCA, the FXR ligand, significantly decreased rCYP7A1 expression (Fig. 3B). The expression of rBSEP, rNTCP and rMRP2 was significantly decreased during control incubation, whereas that of rMRP3 expression was increased (Fig. 4A). 1,25(OH)$_2$D$_3$ did not affect rBSEP, rNTCP, rMRP2 and rMRP3 expression significantly. As expected, CDCA induced rBSEP (1.8-fold induction; $P < 0.05$) and rMRP2 (1.8-fold induction; $P < 0.05$) and decreased rNTCP (0.5-fold decrease; $P < 0.05$) expression without affecting rMRP3 expression (Fig. 4B). Further 1,25(OH)$_2$D$_3$ and CDCA-treated rat liver slices samples were analyzed for the nuclear receptors/transcription factors involved in the regulation of bile acid synthesis and transporters such as rVDR, rFXR, rLXRα, rPXR, rSHP, rHNF1α, HNF4α and rLRH-1. In the control incubated rat liver slices, the expression of rVDR mRNA was significantly elevated (5-fold induction; $P < 0.05$) and the expression of rFXR, rLXRα, rSHP, rHNF1α, and rHNF4α was significantly decreased, whereas rPXR and rLRH-1 mRNA expression was not affected (Fig. 5A and 6A). 1,25(OH)$_2$D$_3$ induced rVDR expression in a dose-dependent manner (2.5-fold induction at 10 nM up to 3.5-fold at 100 nM of 1,25(OH)$_2$D$_3$; $P < 0.05$), rFXR and rPXR was decreased (20% decrease at 200 nM of 1,25(OH)$_2$D$_3$; $P < 0.05$), and rLXRα, rSHP, rHNF1α, rHNF4α and rLRH-1 mRNA expression was not affected (Figs. 5B and 6B). CDCA induced rVDR (2.2-fold induction at 100 μM of CDCA; $P < 0.05$) and rSHP expression (3.0-fold induction at 100 μM of CDCA; $P < 0.05$) (Figs. 5B and 6B). Furthermore, CDCA significantly decreased rFXR, rLXRα, rPXR rHNF1α, HNF4α and rLRH-1 expression (Figs. 5B and 6B).

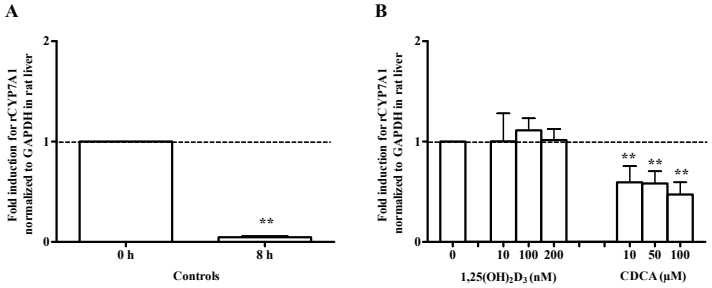

*Figure 3. The expression of rCYP7A1 mRNA in the rat liver slices was unstable during 8 h of incubation (A). Rat liver slices were treated with 1,25(OH)$_2$D$_3$ (10 nM, 100 nM, and 200 nM) and CDCA (10 μM, 50 μM and 100 μM) for 8 h. rCYP7A1 mRNA expression was not affected by 1,25(OH)$_2$D$_3$ but CDCA significantly decreased rCYP7A1 mRNA expression (B). Results are expressed as mean ± S.E.M. of 3-4 rats. "**" indicates $P < 0.001$.*

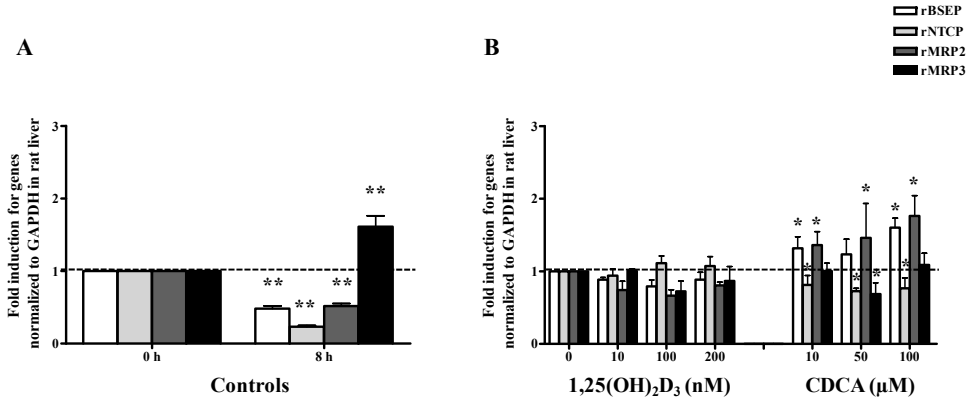

Figure 4. The rBSEP, rNTCP and rMRP2 mRNA expression was significantly decreased whereas rMRP3 expression was significantly increased in the rat liver slices during 8 h of incubation (A). rBSEP, rNTCP, rMRP2 and rMRP3 gene expression was not affected by 1,25(OH)$_2$D$_3$. CDCA, significantly increased rBSEP and rMRP2 gene expression, and significantly decreased rNTCP gene expression, whereas rMRP3 gene expression was not affected (B). Results are expressed as mean ± S.E.M. of 3-4 rats. "" indicates P < 0.05; and "**" indicates P < 0.001.*

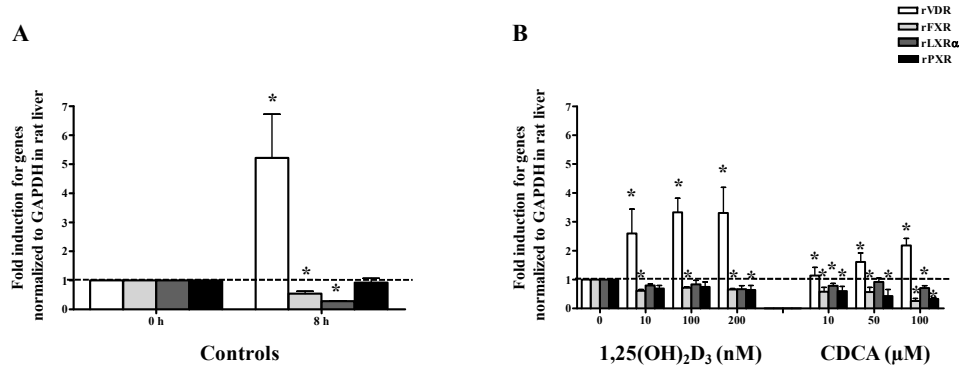

Figure 5. The rat rFXR and rLXRα, was significantly decreased; rVDR gene expression was significantly increased and whereas those of the rat rPXR unaffected in the rat liver slices during 8 h of incubation (A). Incubation with 1,25(OH)$_2$D$_3$ significantly decreased rFXR, rLXRα, and rPXR gene expression; rVDR gene expression was significantly increased; CDCA, significantly increased rVDR gene expression, and significantly decreased rFXR, rLXRα, and rPXR gene expression (B). Results are expressed as mean ± S.E.M. of 3-4 rats. "" indicates P < 0.05; and "**" indicates P < 0.001.*

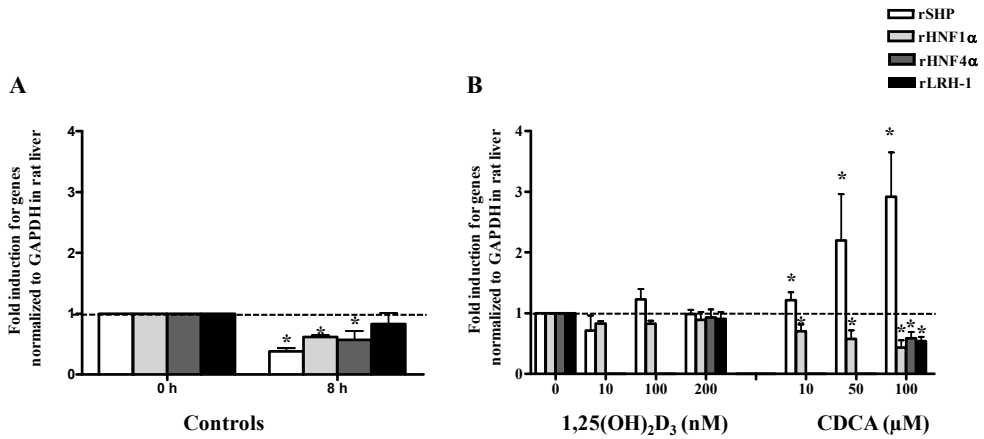

Figure 6. The expression of rSHP, rHNF1α and rHNF4α was significantly decreased whereas rLRH-1 expression was unaffected in the rat liver slices during 8 h of incubation (A). Incubation with 1,25(OH)$_2$D$_3$ did not affect rSHP, rHNF1α, rHNF4α and rLRH1 gene expression and CDCA, significantly increased rSHP and decreased rHNF1α, rHNF4α and rLRH-1 gene expression (B). Results are expressed as mean ± S.E.M. of 3-4 rats. "" indicates P < 0.05; and "**" indicates P < 0.001.* ***Note:*** *rHNF4α and rLRH-1 gene expression was analyzed only in the liver slices treated with 200 nM of 1,25(OH)$_2$D$_3$ and 100 μM of CDCA.*

Effect of VDR and FXR ligands on the expression of the bile acid synthesis enzyme, transporters and nuclear receptors/transcription factors in human liver slices. In human liver slices, CYP7A1 mRNA expression was stable during 24 h of incubation in absence of treatment (control incubation), whereas the expression of BSEP, NTCP and FXR mRNA was decreased, and the expression of MRP2, MRP3, SHP, HNF1α and HNF4α expression was increased (Figs. 7A, 8A, 9A and 10A).

The incubation of human liver slices with 1,25(OH)$_2$D$_3$ significantly decreased CYP7A1 (40% decrease at 200 nM of 1,25(OH)$_2$D$_3$; $P < 0.05$), NTCP (40% decrease at 200 nM of 1,25(OH)$_2$D$_3$; $P < 0.05$) and HNF4α (40% decrease at 200 nM of 1,25(OH)$_2$D$_3$; $P < 0.05$) expression (Figs. 7B, 8B and 10B) but did not affect BSEP, MRP2, MRP3, VDR, SHP, LXRα and HNF1α expression (Fig. 7B, 8B, 9B and 10B). 1,25(OH)$_2$D$_3$ slightly but significantly decreased FXR (Fig. 9B). CDCA significantly decreased CYP7A1 (60% decrease at 100 μM of CDCA; $P < 0.05$) and slightly decreased the NTCP expression (20% decrease at 100 μM of CDCA; $P < 0.05$). The expression of BSEP (3.2-fold induction at 100 μM of CDCA; $P < 0.05$), MRP3 (1.8-fold increase at 100 μM of CDCA; $P < 0.05$) and SHP mRNA (3.2-fold increase, $P< 0.05$) (Figs 9B and 10B) was induced by CDCA, but MRP2 expression was unaffected (Fig. 8B). One of the human liver donors was found to be deficient in MRP3 mRNA expression and did not show up-regulation of MRP3 with CDCA despite that other FXR target genes such as CYP7A1 repression were decreased. LXRα, and HNF4α expression was not affected by CDCA (Figs. 9B and 10B) and CDCA induced PXR expression in three out of five livers, whereas the other two livers showed a decrease in PXR expression (Fig. 9B).

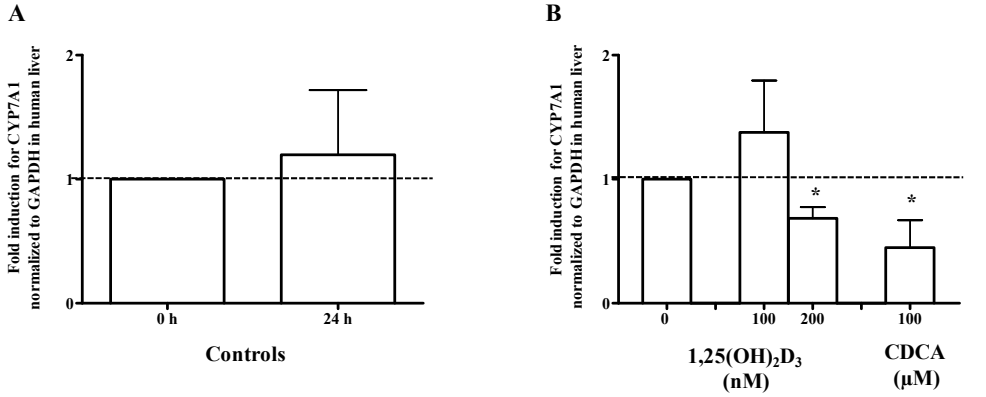

Figure 7. The mRNA expression of CYP7A1 in the human liver slices was stable during 24 h of incubation (A). CYP7A1 expression was significantly decreased by 1,25(OH)$_2$D$_3$ and CDCA (B). Results are expressed as mean ± S.E.M. of 4-5 human liver donors. "" indicates P < 0.05.*

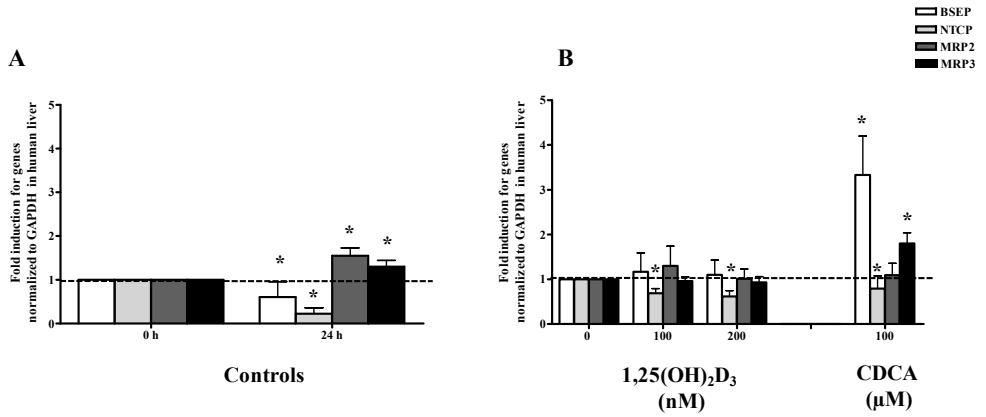

Figure 8. Human BSEP, NTCP was decreased and MRP2 and MRP3 gene expression was significantly increased in the human liver slices during 24 h of incubation (A). 1,25(OH)$_2$D$_3$ significantly decreased NTCP expression; BSEP, MRP2 and MRP3 gene expression was not affected; CDCA significantly increased BSEP and MRP3 gene expression, and significantly decreased NTCP gene expression, whereas MRP2 gene expression was not affected (B). Results are expressed as mean ± S.E.M. of 4-5 human liver donors. "" indicates P < 0.05.*

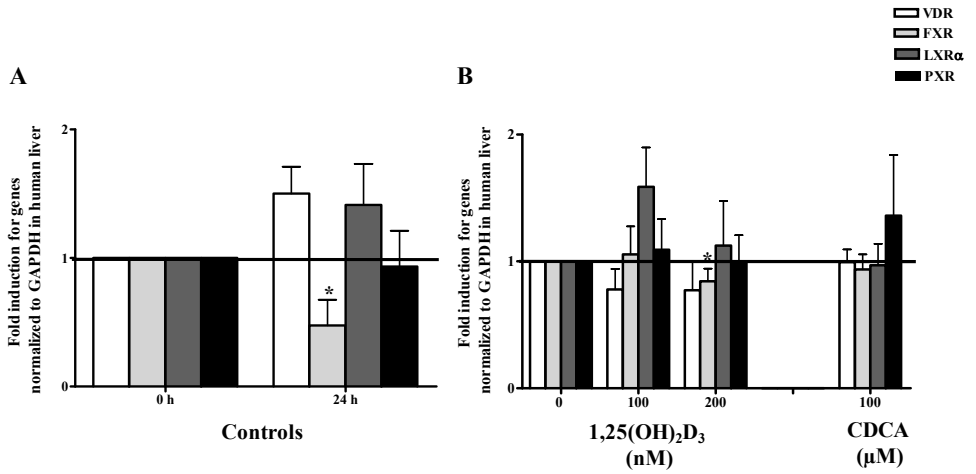

Figure 9. Human VDR, LXRα, and PXR gene expression was not affected but FXR expression was significantly decreased in the human liver slices during 24 h of incubation (A). 1,25(OH)$_2$D$_3$ did not affect VDR, LXRα, FXR and PXR gene expression; CDCA did not affect VDR, FXR, LXRα and PXR gene expression (B). Human liver slices were treated with 1,25(OH)$_2$D$_3$ (10 nM, 100 nM, and 200 nM) and CDCA (10 μM, 50 μM and 100 μM) for 24 h. Results are expressed as mean ± S.E.M. of 4-5 human liver donors. "" indicates P < 0.05.*

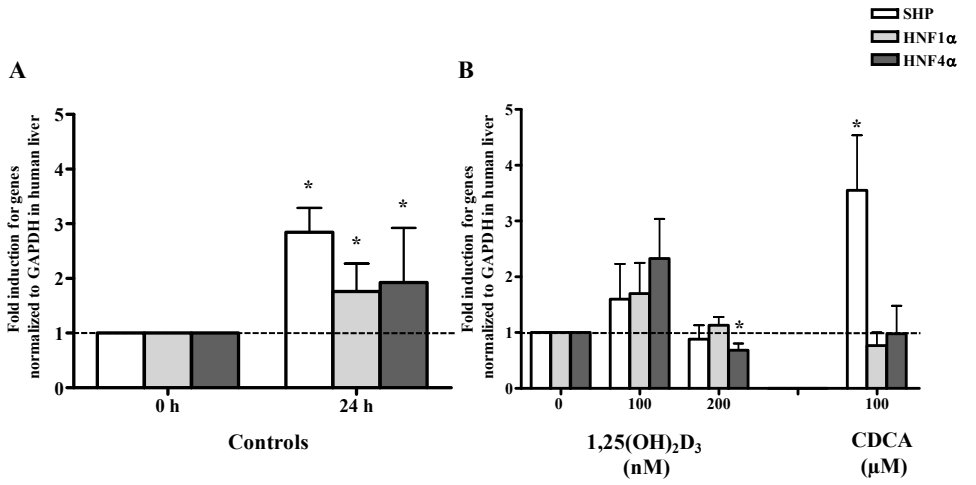

Figure 10. Human HNF1α and HNF4α gene expression was not affected but SHP was significantly increased in the human liver slices during 24 h of incubation (A). 1,25(OH)$_2$D$_3$ significantly decreased HNF4α gene expression whereas SHP and HNF1α gene expression was not affected; CDCA significantly induced SHP expression and did not affect HNF1α and HNF4α gene expression (B). Results are expressed as mean ± S.E.M. of 4-5 human liver donors. "" indicates P < 0.05.*

Discussion

Recently, the vitamin D receptor (VDR) was found to play a role in the regulation of detoxification enzymes and transporters in various tissues along with the pregnane X

receptor (PXR), farnesoid X receptor (FXR), and constitutive androstane receptor (CAR), (25, 29, 40, 41). Consistent with the earlier reports (12, 13), Khan et al (25) and Chow et al. (7) showed higher VDR mRNA expression in rat intestinal epithelial cells compared to that in liver tissue, though varying along the length of the intestine, with the highest expression in colon. In agreement with the VDR distribution in rat intestine and liver, VDR target genes such as CYP3A1 and ASBT were highly responsive in the intestine compared to the liver (5, 25). However the gradient of induction of genes along the length of the rat intestine *in vivo* was not related to the gradient in the expression of VDR. Therefore, it can be argued that, not only the VDR expression level but the level of exposure of the tissue to the ligand, $1,25(OH)_2D_3$, after its intraperitoneal injection, *in vivo*, the intracellular disposition of the ligand, and the presence of co-activators may play a role (7, 25). However, it was observed that Sprague-Dawley rats treated with $1,25(OH)_2D_3$ were associated with altered expression of bile synthesis proteins and transporters in the liver (7) despite the lack of localization of VDR in parenchymal cells (13) and confirmed in the present study with the Wistar rats (Figs. 1C and F). Thus, we hypothesized that changes in the expression of proteins involved in bile acid synthesis and disposition after intraperitoneal administration of $1,25(OH)_2D_3$ in the rat liver (7) might be secondary to the increased bile acid flux due to increased bile acid absorption.

To test this hypothesis, we treated rat and human liver slices, an *ex-vivo* model with adequate representation of all the cell types of the liver, with the VDR ligand, $1,25(OH)_2D_3$, and also with the primary bile acid, CDCA, as the FXR ligand. $1,25(OH)_2D_3$ did not affect the expression of the rCYP7A1, the enzyme for bile acid synthesis nor the sinusoidal transporters, rNTCP and rMRP3, and the canalicular transporters, rBSEP and rMRP2 (Fig. 4B). Furthermore, the expression of the nuclear receptors known to regulate these proteins, rSHP, rHNF1α, rHNF4α, rLXRα and rLRH-1, remained unchanged (Figs. 5B and 6B). In contrast, CDCA significantly decreased rCYP7A1 expression, with simultaneous induction of rSHP and repression of rHNF1α, rHNF4α, rLXRα and rLRH-1, as expected for the FXR ligand (Figs. 5B and 6B). Moreover, CDCA induced the rBSEP and rMRP2 expression, decreased the rNTCP expression but not that for rMRP3. These results are consistent with the earlier findings (2, 10) where CDCA was implicated in the feedback inhibition of bile acid synthesis by binding to the FXR and decreasing the expression of CYP7A1 and NTCP by inducing SHP expression. Induction of rMRP2 by CDCA had not been reported before in the rat liver. In addition to the SHP-dependent pathway, CYP7A1 may be further affected by other SHP-independent pathways, such as rHNF4α repression (1, 14) or decreased rLXRα and rLRH-1, which are essential for the expression of rCYP7A1 (9, 26). In addition, CDCA also decreased the expression of rFXR and rPXR (Fig. 5B), an observation that had not been reported before for the rat. Thus, apart from decreasing the expression of CYP7A1, the activation of FXR by CDCA further decreased FXR expression, probably by a feedback loop mediated by decreased expression of HNF1α (Fig. 6B), which is required for its basal expression (28). The CDCA-dependent repression of PXR is consistent with that found in human hepatocytes and in mice mediated

by SHP induction (34), but is in contrast to the increased expression of PXR found in mice fed with CDCA and GW4064 by Jung et al. (23).

These contrasting results of VDR and FXR ligands on the regulation of bile acid homeostasis proteins in the rat liver support the hypothesis that the decrease in CYP7A1 protein and activity by 1,25(OH)$_2$D$_3$ in Sprague-Dawley rats that occurred with the simultaneous induction of SHP reported by (Chow et al., 2009, accepted), was secondary to the increased bile acid absorption, subsequent to the induction of ileal rASBT. The induction of FXR expression in these Sprague-Dawley rats is in contrast to our in vitro results with Wistar rat liver slices treated with CDCA and 1,25(OH)$_2$D$_3$, where a significant decrease in FXR mRNA was found. The composite results are in accordance with the confinement of VDR in the bile duct epithelial cells and the compartmentalization of bile acid synthesis proteins in hepatocytes, as evident from our immunohistochemistry data as well as from earlier reports (13). However, the 1,25(OH)$_2$D$_3$ mediated repression of rFXR mRNA, which is predominantly localized in hepatocytes remains to be explained, but might be attributed to paracrine effects of 1,25(OH)$_2$D$_3$, or might be the result of an interaction of 1,25(OH)$_2$D$_3$ with an as yet unknown NR.

Both, 1,25(OH)$_2$D$_3$ and CDCA significantly up-regulated the VDR expression in a dose-dependent fashion in the rat liver (Fig. 5B). Our results are the first to show that the VDR is positively regulated by 1,25(OH)$_2$D$_3$ as well as by CDCA in the rat liver slices. The rat and mouse intestine and kidney VDR were shown to be regulated by 1,25(OH)$_2$D$_3$ in vivo (18, 39). It was later shown that 1,25(OH)$_2$D$_3$ regulated the expression of VDR in a organ specific manner in mice (18). The effects of CDCA on the expression of VDR were not reported before and further studies are needed to elucidate the molecular mechanism of CDCA mediated induction of VDR in rat liver.

Subsequent to studying the effects of VDR and FXR ligands in rat liver slices, we investigated the effects of 1,25(OH)$_2$D$_3$ and CDCA in human liver slices. In the human liver, VDR expression was found both in the hepatocytes as well as in the nonparenchymal cells (BEC) (Figs. 2D, E and F), consistent with earlier reports (13). The expression of VDR in the human livers was also confirmed at the level of mRNA, similar to the earlier report in the human hepatocytes (11). The expression of VDR mRNA in human liver donors showed a high variation with levels of VDR mRNA which were undetectable with the primer set used in three out of five human liver donors as reported earlier by us (25), and summarized in our recent report (chapter 7). In contrast to what was found in the rat liver slices, 1,25(OH)$_2$D$_3$ significantly decreased the expression of CYP7A1 in human liver slices (Fig. 7B). Repression of CYP7A1 was also found after treatment with CDCA, which can be explained by the induction of SHP and the subsequent repression of HNF1α and HNF4α (Fig. 10B), mediated by the FXR-SHP-LRH-1 cascade (15). In contrast to CDCA, 1,25(OH)$_2$D$_3$ significantly decreased the expression of HNF4α without affecting SHP and HNF1α expression. The CYP7A1 repression by 1,25(OH)$_2$D$_3$ is congruent with the recent

report by Han et al. (17) who showed a decrease in the CYP7A1 expression in human hepatocytes and HepG2 cells treated with $1,25(OH)_2D_3$. The VDR dependent CYP7A1 repression was reported to be mediated by a decrease in the association of co-activators and an increase in the association of co-repressors with HNF4α on the CYP7A1 promoter by the interaction of VDR with the HNF4α protein (17). Our results show, for the first time, that $1,25(OH)_2D_3$ decreased the expression of HNF4α in human liver slices, which may contribute to a further decrease in expression of CYP7A1 (1). $1,25(OH)_2D_3$ significantly decreased NTCP expression in human liver slices, without affecting the expression of BSEP, MRP2 and MRP3. Similar to CYP7A1 regulation, NTCP is also regulated by HNF4α by binding to the promoter (14). Our results are the first to show that human NTCP is significantly decreased by the $1,25(OH)_2D_3$ and further studies are needed to elucidate whether this is also mediated via HNF4α down regulation. Similar to rat liver slices, CDCA significantly induced BSEP and decreased NTCP expression (Fig. 8B) (2, 10). The slight decrease in NTCP expression in human livers with CDCA was not found in our earlier studies (22). In contrast to the results of rat liver slices, CDCA induced MRP3 but not MRP2 in human liver slices (Fig. 8B) which is in line with the earlier reports by Inokuchi et al. (21), who characterized a bile acid response element in the human MRP3 promoter.

In conclusion, the expression of CYP7A1 was decreased by the VDR ligand, $1,25(OH)_2D_3$, in the human but not the rat liver, whereas the FXR ligand, CDCA decreased CYP7A1 expression both in human and rat livers. This species difference in CYP7A1 regulation is consistent with the differential localization of VDR in the rat liver (exclusively in non parenchymal cells) and human liver (non-parenchymal cells and hepatocytes). This is further supported by the lack of effect of $1,25(OH)_2D_3$ on any of the genes involved in bile acid transport in rat liver. By contrast, a VDR mediated down-regulation of HNF4α and NTCP expression was found in the human liver. Further studies are needed to elucidate the implication of VDR mediated regulation of bile acid synthesis and transport proteins in human 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).

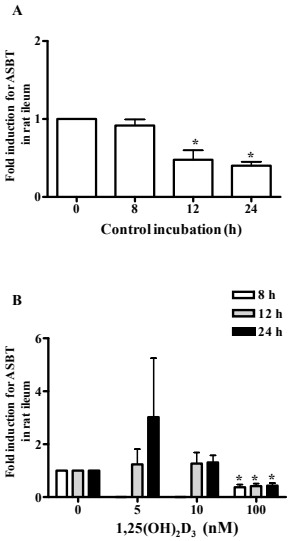

*Figure 1. Rat ileum slices were exposed to incubation with vehicle (A), and $1,25(OH)_2D_3$ (5-100 nM) (B) for 8 h, 12 h and 24 h, after which total RNA was isolated and mRNA expression of ASBT was evaluated by qRT-PCR. Results were expressed as fold-induction after normalizing with β-actin, and compared 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).

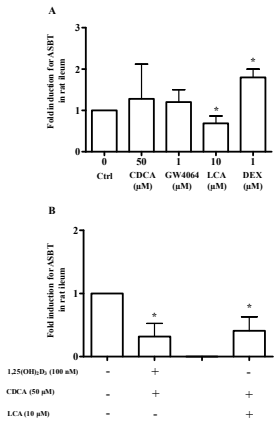

*Figure 2. Slices from rat ileum were exposed to CDCA (50 μM), LCA (5-10 μM), GW4064 (1 μM) and DEX (1 μM) for 8 h (A), $1,25(OH)_2D_3$ (100 nM) + 50 μM of CDCA and LC (10 μM) + 50 μM of CDCA for 8 h (B) after which total RNA was isolated and mRNA expression of ASBT was evaluated by qRT-PCR. Results 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 $P < 0.05$.*

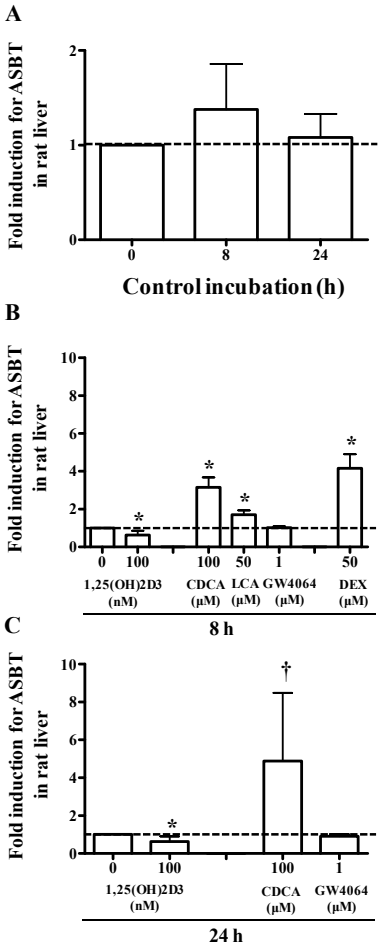

*Figure 3. Rat liver slices were exposed to incubation with vehicle for 0 h, 8 h and 24 h (A), 1,25(OH)$_2$D$_3$ (100 nM), CDCA (50 μM), LCA (50 μM), GW4064 (1 μM) and DEX (50 μM) for 8 h (B) and 1,25(OH)$_2$D$_3$ (100 nM), CDCA (50 μM) and GW4064 (1 μM) for 24 h (C), after which total RNA was isolated 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).

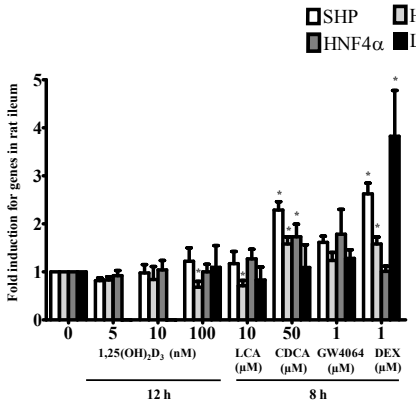

*Figure 4. Slices from rat ileum were exposed to 1,25(OH)$_2$D$_3$ (5-100 nM), CDCA (50 μM), LCA (10 μM), GW4064 (1 μM) and DEX (1 μM) for 8 h or 12 h after which total RNA was isolated and mRNA expression of SHP, HNF1α, HNF4α and LRH-1 was evaluated by qRT-PCR. Results were expressed as fold-induction 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).

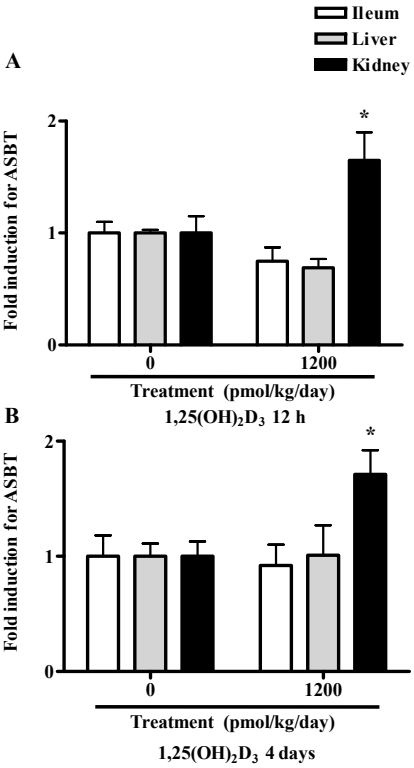

*Figure 5. Wistar rats were treated with with 1,25(OH)$_2$D$_3$ (1200 pmol/kg/day) for 12 h (A) and 4 days (B) by intraperitoneal injection. After 12 h or 24 h of the last dose administered, rats were sacrificed and total RNA was isolated from ileum, liver and kidneys and mRNA expression of ASBT in ileum, 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)

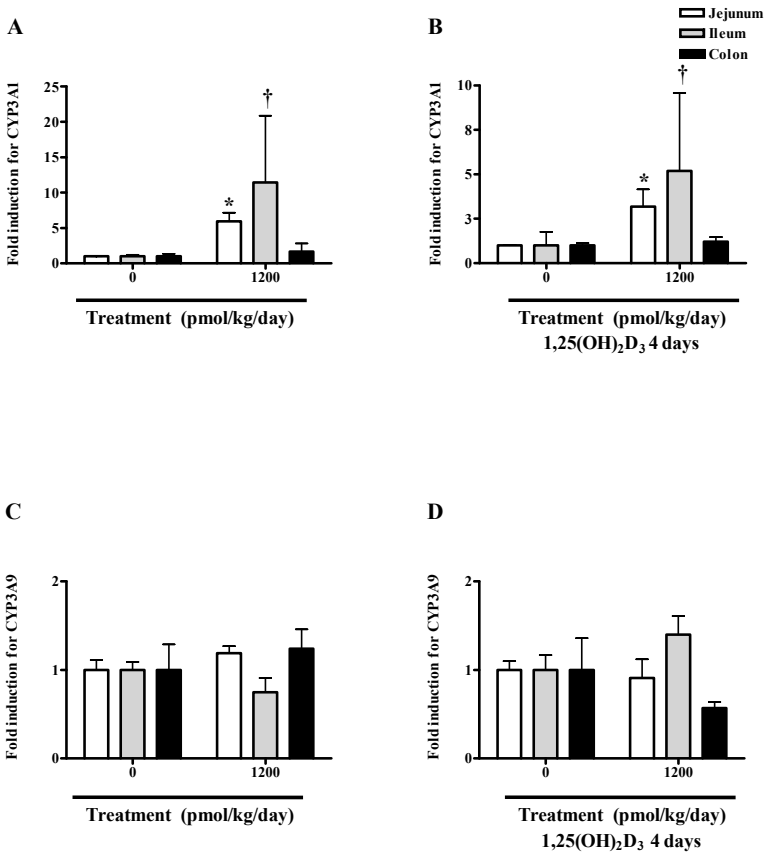

*Figure 6. Wistar rats were treated with with $1,25(OH)_2D_3$ (1200 pmol/kg/day) for 12 h and 4 days by intraperitoneal injection. After 12 h or 24 h of the last dose administered, rats were sacrificed and total RNA was isolated from jejunum, ileum and colon mRNA expression of CYP3A1 (A and B) and CYP3A9 (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).

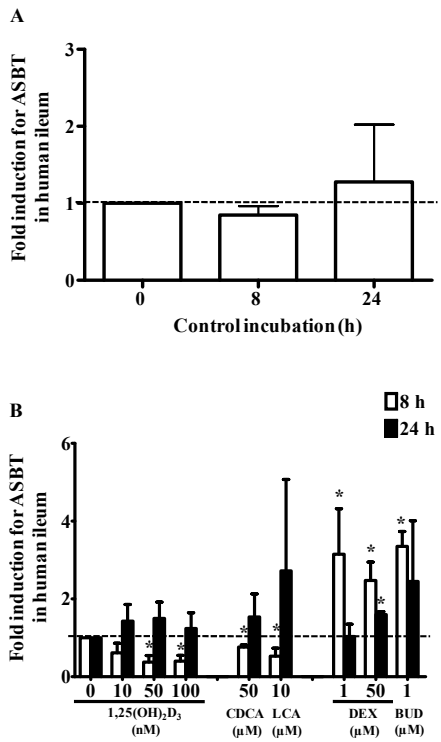

*Figure 7. Human ileum slices were exposed to control incubation (A)1,25(OH)$_2$D$_3$ (10-100 nM), CDCA (50 µM), LCA (10 µM), DEX (1-50 µM) and BUD (1 µM) (B) for 8 h and 24 h, after which total RNA was isolated and mRNA expression of ASBT was evaluated by qRT-PCR. The results were expressed as fold-induction 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.*

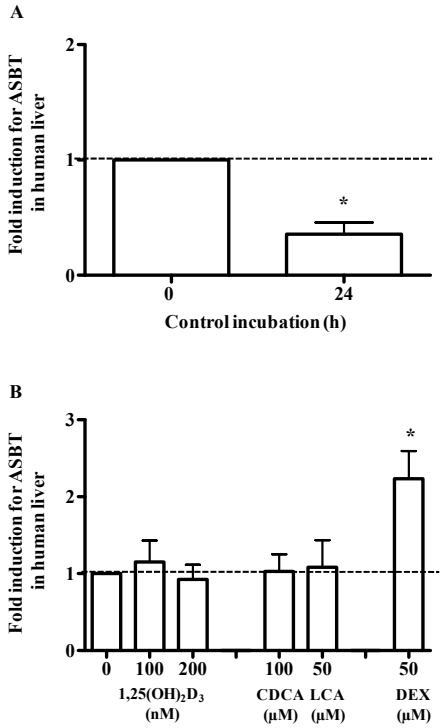

*Figure 8. Human liver slices were exposed to control incubation (A), 1,25(OH)$_2$D$_3$ (100-200 nM), CDCA (50 µM) and LCA (10 µM), DEX (50 µM) (B) for 24 h, after which total RNA was isolated and mRNA expression of ASBT was evaluated by qRT-PCR. Results were expressed as fold-induction after normalizing 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).

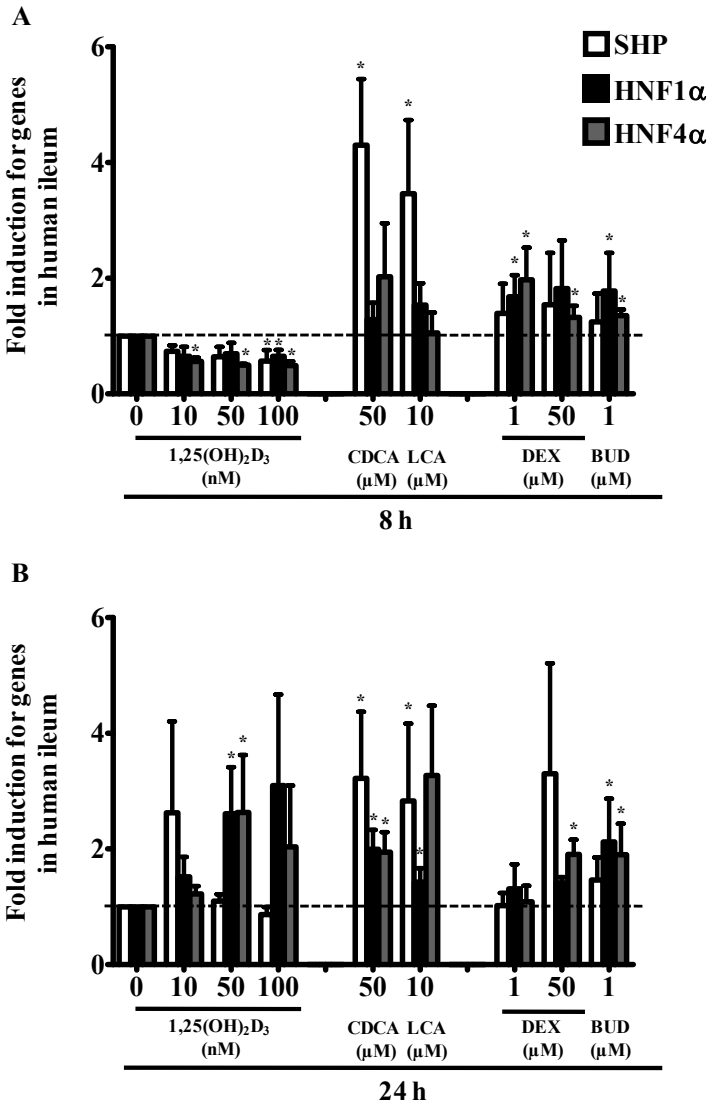

*Figure 9. Human ileum slices were exposed to 1,25(OH)$_2$D$_3$ (10-100 nM), CDCA (50 μM), LCA (10 μM), DEX (1-50 μM) and BUD (1 μM) (B)for 8 h (A) and 24 h (B), after which total RNA was isolated and mRNA expression of SHP, HNF1α and HNF4α was evaluated by qRT-PCR. Results were expressed as fold-induction 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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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.

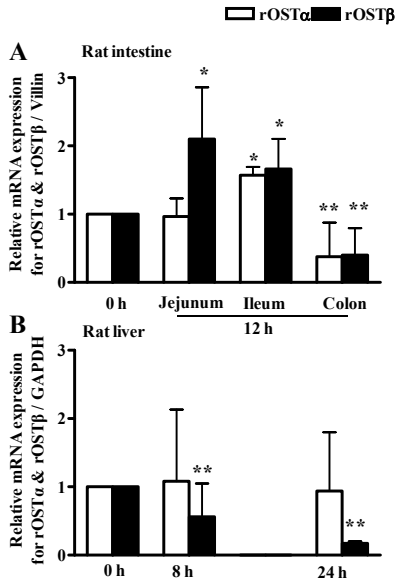

Figure 1. mRNA expression of rASBT, rOSTα and rOSTβ transporters relative to villin expression in intestinal tissue (jejunum, ileum and colon) of the Wistar rat. The average threshold cycles (C_T) for rASBT in jejunum was 29, in ileum 23 and in colon 30.The average C_T for rOSTα and rOSTβ in jejunum 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

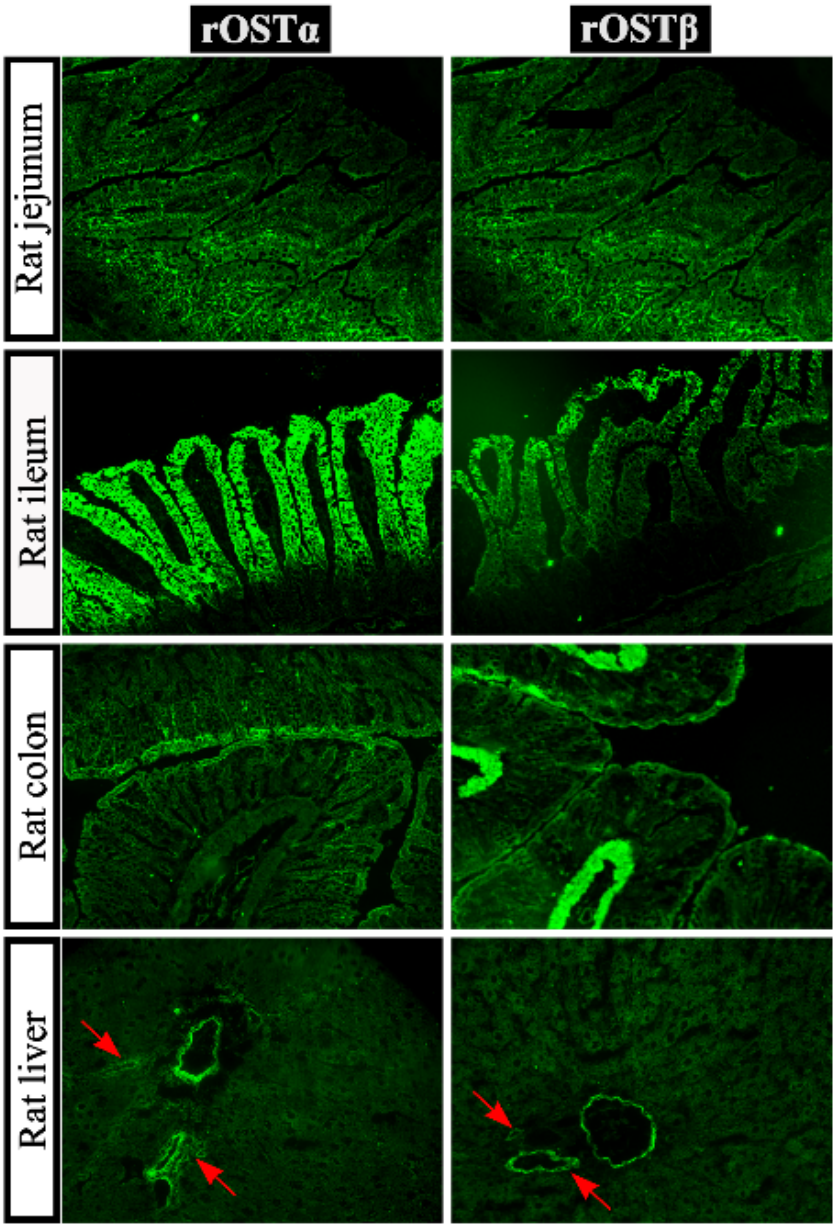

Figure 2. Indirect immunofluorescence showed that rOSTα and rOSTβ were detected at the basolateral surface of the enterocytes in the jejunum, ileum and colon of the Wistar rat. In the liver, rOSTα and rOSTβ proteins are predominantly localized in the bile duct epithelial cells and a low expression was 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).

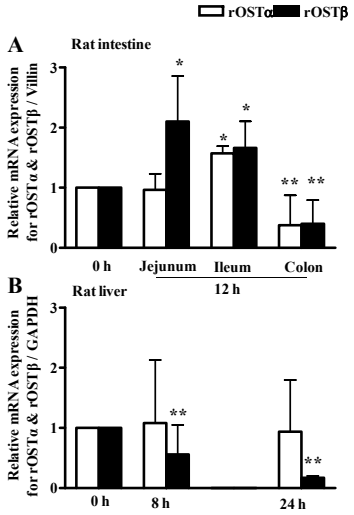

Figure 3. The effect of incubation at 37ºC on rat jejunum, ileum, colon (12 h) and liver slices (8 h and 24 h) on the expression of rOSTα and rOSTβ genes. The mRNA expression of rOSTα and rOSTβ genes relative to villin (intestine) and GAPDH (liver) was quantified by real-time PCR and expressed with respect 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.

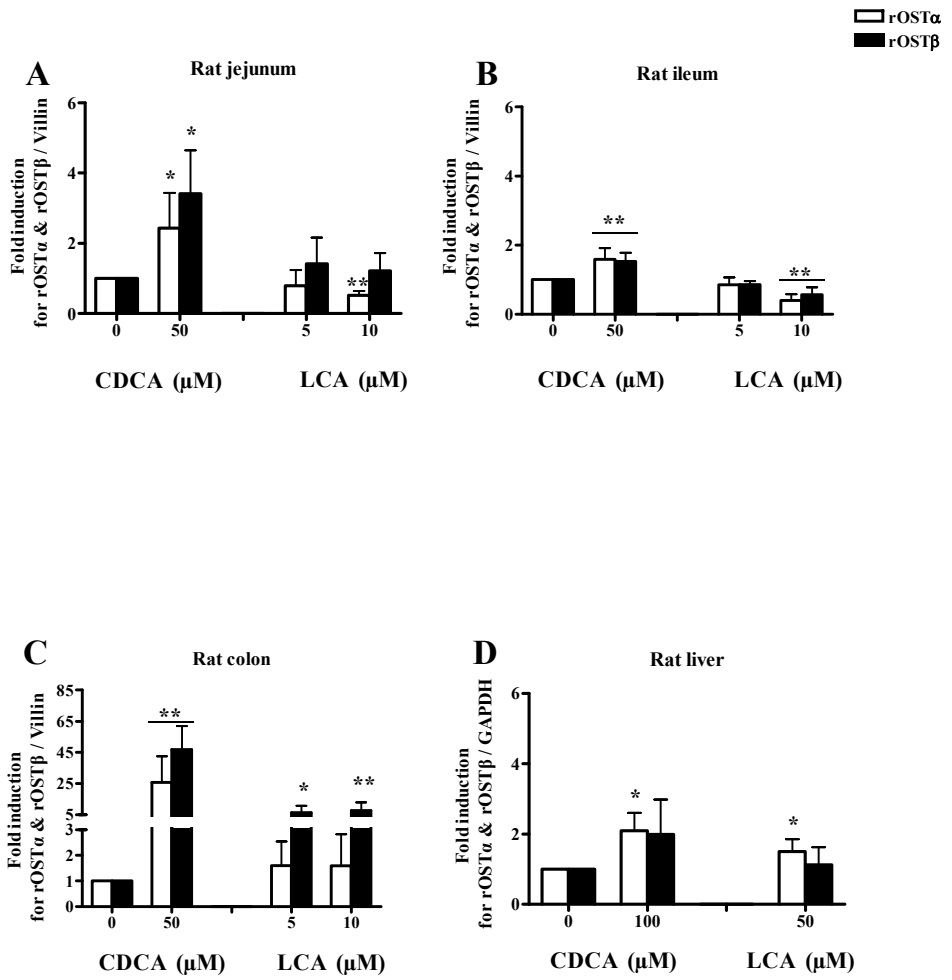

Figure 4. rOSTα and rOSTβ genes are induced by FXR ligands. Rat intestine slices (jejunum (A), ileum (B) and colon (C)) were treated with CDCA (50 μM) and LCA (5 μM and 10 μM) for 12 h, and liver slices (D) were treated with 100 μM of CDCA and 50 μM of LCA for 24h. The mRNA expression of rOSTα and rOSTβ 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).

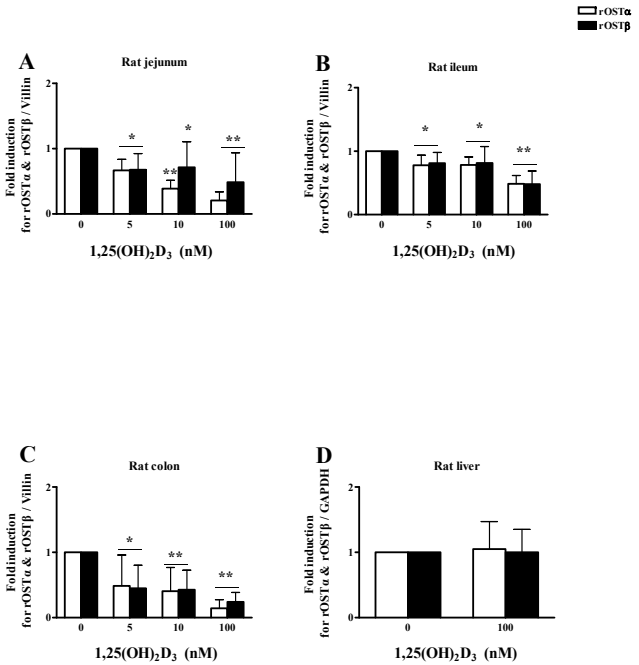

Figure 5. The VDR ligand, $1,25(OH)_2D_3$ decreases the expression of rat rOSTα and rOSTβ genes in jejunum (A), ileum (B), colon (C) and liver (D) slices. Rat jejunum, ileum and colon slices were treated with 5 nM, 10 nM and 100 nM of $1,25(OH)_2D_3$ for 12 h. Rat liver slices were treated with 100 nM 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 $P < 0.001$*

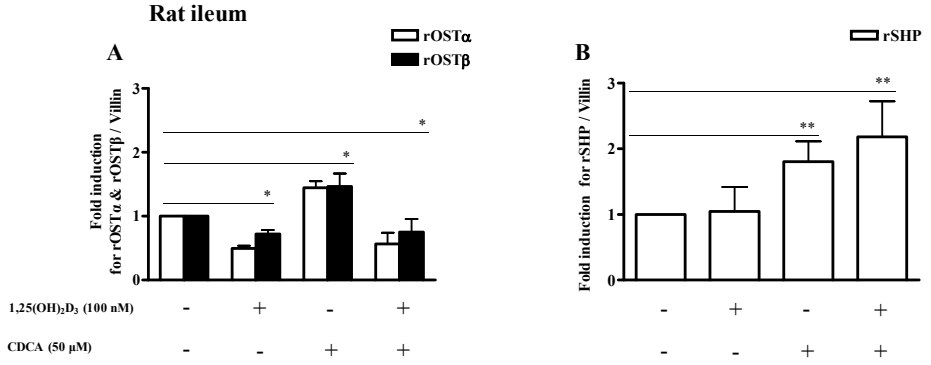

Figure 6. The VDR ligand, 1,25(OH)$_2$D$_3$, decreases the expression of rat rOSTα and rOSTβ genes in ileum, also in the presence of FXR ligand, CDCA (A). 1,25(OH)$_2$D$_3$ did not affect the induction of SHP by CDCA in ileum slices (B). Rat ileum slices were treated with 100 nM of 1,25(OH)$_2$D$_3$, 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.

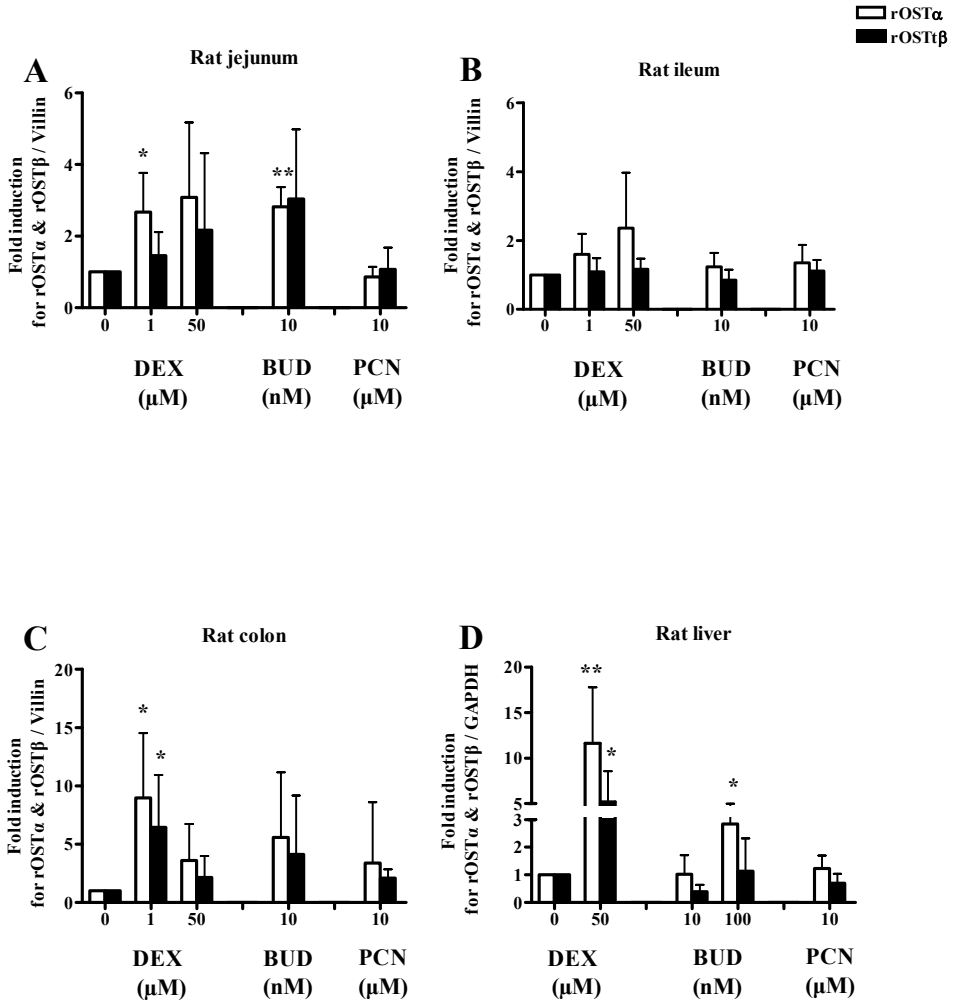

Figure 7. The GR ligands, dexamethasone (DEX) and budesonide (BUD) but not the PXR ligand, pregnane 16-α carbonitrile (PCN) induce the expression of rat rOSTα and rOSTβ genes in jejunum (A), colon (C), but not in ileum (B) and liver (D) slices. Rat jejunum, ileum and colon slices were treated with 1 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.

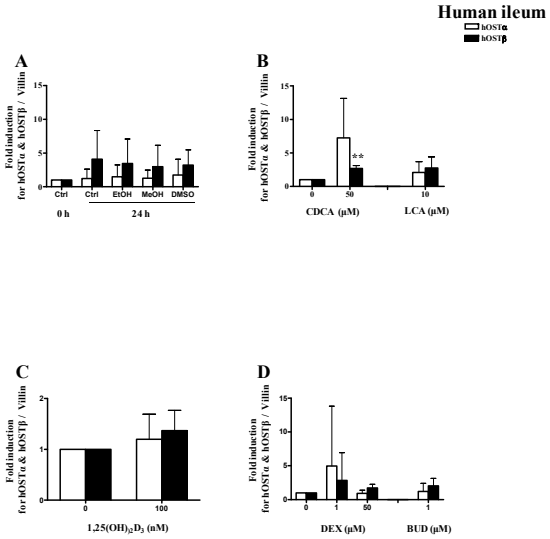

Figure 8. The effect of incubation time (24 h) and the solvent controls on the expression of human organic solute transporter, hOSTα and hOSTβ genes in human ileum slices (A). The mRNA expression of hOST α and hOST β genes relative to villin was quantified by real-time PCR and expressed with respect 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).

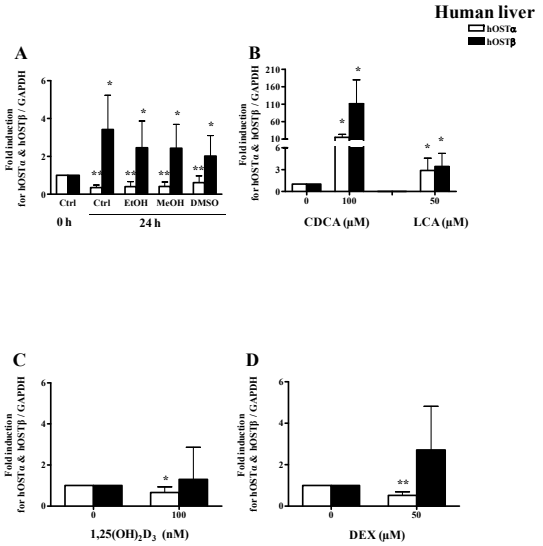

Figure 9. The effect of incubation time (24 h) and the solvent controls on the expression of human organic solute transporter (hOST)α and hOSTβ genes in human liver slices (A). The mRNA expression of hOSTα and hOSTβ genes relative to GAPDH were quantified by real-time PCR and expressed with respect to 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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Chapter 7

Regulation of Vitamin D Receptor (VDR) Expression in Rat and Human intestine and Liver – Consequences for bile acid detoxification

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Abstract

The vitamin D receptor (VDR), activated by its ligands, 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and LCA, regulates the expression of drug metabolizing enzymes and transporters in intestine and liver, but the regulation of VDR expression in intestine and liver is incompletely understood. We studied the regulation of VDR expression at the level of mRNA by ligands for VDR, farnesoid X receptor (FXR), glucocorticoid receptor (GR) and protein kinase C α (PKCα) in rat and human ileum and liver using precision-cut slices. Further, we investigated a possible interaction of chenodeoxycholic acid (CDCA) with LCA and $1,25(OH)_2D_3$ on the regulation of VDR and CYP3A isozymes in rat ileum and liver. The mRNA expression of VDR, CYP3A1 and CYP3A2 was evaluated by qRT-PCR. The expression of VDR mRNA was significantly induced by $1,25(OH)_2D_3$ in rat ileum and liver slices, and human ileum but not in human liver slices. CDCA, but not LCA and the synthetic FXR ligand, GW4064 induced VDR mRNA expression in rat ileum and liver slices. The PKCα activator, phorbol-12-myristate-13-acetate (PMA) induced the expression of VDR in the rat liver, and the induction of VDR by $1,25(OH)_2D_3$ and CDCA was inhibited by the PKC inhibitor, bisindolyl maleimide (Bis I). The GR.ligand, dexamethasone decreased the VDR expression in rat ileum and liver. These results show that the expression of VDR in the rat liver is likely to be regulated by GR and PKCα but not by FXR or VDR activation. The mechanism of induction in the rat and human ileum remains to be elucidated. The VDR mediated induction of CYP3A isozymes in the rat ileum by $1,25(OH)_2D_3$ and LCA was strongly reduced in the presence of CDCA despite the higher VDR expression. Thus, CDCA might potentiate the toxicity of LCA by inhibiting its metabolism and this might be one of the possible mechanisms of increased incidence of colon cancer in populations on high fat diet.

Keywords: Vitamin D receptor**,** cytochrome P450, induction, intestinal slices, liver slices, lithocholic acid

Introduction

The vitamin D receptor (VDR) is a member of the steroid-thyroid hormone nuclear receptor family, NR1I1 and it is expressed predominantly in organs that play an important role in calcium and phosphorous homeostasis such as intestine, kidney, bone and parathyroid glands. VDR is also expressed at a lower level in other tissues, such as liver, muscle and prostate glands (45). VDR regulates the expression of genes involved in calcium and phosphorous metabolism in intestine, kidney and bone (6, 11). Moreover, VDR exhibits a significant homology with PXR and CAR (26) and is reported to modulate xenobiotic metabolism and transport (1, 5, 18, 28, 41, 47). VDR is activated by 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and the toxic bile acid, lithocholic acid (LCA), and its metabolite 3α-keto cholastanic acid (3-KCA), hence VDR is considered as an intestinal bile acid sensor (25). The ligand-activated VDR heterodimerizes with retinoic acid X receptor α (RXRα; NR2B1), and binds to the VDR response elements (VDRE) in the promoters of target genes (35, 39, 41, 48), which are characterized as direct-repeats separated by three nucleotide base pairs (DR3) and everted-repeats with two different direct-repeats separated by six-nucleotide base pairs (ER6) (11, 40).

The regulation of VDR expression is incompletely understood. The VDR ligand, $1,25(OH)_2D_3$ is reported to up-regulate VDR mRNA expression in mouse fibroblasts, pig kidney cells (LLC-PK1) and human promyelocytic leukemic cells (HL-60) (3, 23, 29). Strom et al. (38) showed that the treatment of vitamin D-deficient Sprague-Dawley rats with $1,25(OH)_2D_3$ induced VDR mRNA and protein expression in the duodenum within 24 h. Wiese et al (46) found no changes in the mRNA of VDR in mouse fibroblasts *in vitro* and *in vivo* upon treatment with $1,25(OH)_2D_3$ in intestinal epithelial cells of vitamin D-deficient Sprague-Dawley rats but reported an increase in the half-life of VDR protein. Recently, Healy et al. (12) reported the induction of VDR mRNA in kidneys but not in the duodenum of vitamin-D deficient mice treated with $1,25(OH)_2D_3$ in the presence of a supplementary calcium diet. Furthermore, glucocorticoids are reported to down regulate the VDR expression in mouse duodenum and up regulated in rat duodenum (13, 14). Protein kinase C α (PKCα) activators such as phorbol-12-myristate-13-acetate (PMA) were reported to up regulate VDR expression in rat osteosarcoma cells (37). However, these results are in contrast to the earlier reports showing a decrease in the expression of VDR in rat osteosarcoma cells by PMA (20, 44). VDR is also up-regulated by parathyroid hormone (PTH) through the activated protein kinase A (PKA) pathway in mouse osteoblast and osteosarcoma cell lines (19). In summary, this data suggests that VDR might be regulated at the level of mRNA and protein in rat, mouse and man by glucocorticoids, PKCα and $1,25(OH)_2D_3$, but the regulation seems to be species and tissue specific (8).

To obtain more insight in the regulation of VDR mRNA in the human and rat liver and intestine, we performed a systematic study to investigate the direct effects of ligands for VDR ($1,25(OH)_2D_3$ and LCA), FXR (CDCA and GW4064), GR (dexamethasone

(DEX)) as well as those of PKCα activator, PMA on the VDR mRNA expression by using precision-cut slices of rat and human ileum and liver. This *ex-vivo* model was previously shown to be an adequate model to study the regulation of genes of interest by ligands for several NR mediated pathways in the liver (10, 16, 32) and the intestine (18, 27, 42). Moreover, *in vivo*, ligands for several NR's are present simultaneously in the intestine and the portal blood, for instance CDCA, LCA and $1,25(OH)_2D_3$, we investigated a possible interaction of CDCA with LCA and $1,25(OH)_2D_3$ on the regulation of VDR and CYP3A isozymes in rat ileum and liver. Subsequently, we compared the *in vitro* results with those obtained *in vivo* by intraperitoneal treatment (ip) of Wistar rats with $1,25(OH)_2D_3$

Materials and methods

Male Wistar (HsdCpb:WU) rats weighing about 230 - 250 g were purchased from Harlan (Horst, The Netherlands) and were allowed to acclimatize in a temperature and humidity controlled room on a 12-h light/dark cycle with food and tap water *ad libitum* for 7 days before experimentation. Pieces of human liver and ileum tissue were obtained as surgical waste from the University Medical Center Groningen with 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) was purchased from Calbiochem, San Diego, California, dexamethasone was from Genfarma bv, Maarssen. Ethanol, methanol, DMSO, polymethyl sulfonyl flouride (PMSF), and dithiothreitol (DTT) 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). GW4064 was purchased from Tocris Bioscience (Bristol, UK). D-Glucose and HEPES were procured from ICN Biomedicals, Inc. (Eschwege, Germany). Low gelling temperature agarose, phorbol-12-myristate-13-acetate (PMA) 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), 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. The bisindolyl maleimide I (Bis I) is a generous gift from Professor Dr. H. Meurs, Department of Molecular Pharmacology, University of Groningen. All primers were purchased from Sigma Genosys. All reagents and materials used were of the highest purity that was 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 University Medical Center, Groningen.

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. (33). 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 by Khan et. al. (18). 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 earlier published methods (4, 18, 33, 43).

Induction Studies

Incubation of rat and human intestine slices. 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$(OH)_2D_3$ (5 - 100 nM), CDCA (50 µM), LCA (5 - 10 µM), DEX (1 - 50 µM), GW4064 (1 µM) and PMA (1.6 µM) added as a 100-times concentrated stock solution in ethanol (1,25$(OH)_2D_3$), methanol (CDCA and LCA) or DMSO (DEX, PMA and GW4064). Rat intestinal slices were incubated for 8 h or 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; as villin expression was stable up to 24 h.

Rat ileum slices were also incubated with either 1,25$(OH)_2D_3$ (100 nM) or LCA (10 µM) in the absence or presence of CDCA (1, 30 or 50 µM). Control slices were incubated in Williams medium E (supplemented with D-Glucose and gentamicin sulfate) with 1% ethanol, methanol, DMSO and ethanol or methanol + 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. The samples were

stored at – 80° C until RNA isolation. These experiments were replicated in 3 to 5 rats and 3 to 5 human ileum donors.

Incubation of rat and human liver slices. Rat and human 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. Rat liver slices were incubated with $1,25(OH)_2D_3$ (100 nM), CDCA (50 μM and 100 μM), LCA (50 μM), DEX (50 μM) GW4064 (1 μM) and PMA (1.6 μM) added as a 100-fold concentrated stock solution in ethanol (for $1,25(OH)_2D_3$), methanol (for CDCA and LCA) or DMSO (for DEX, PMA and GW4064). Human liver slices were incubated with $1,25(OH)_2D_3$ (100 - 200 nM), CDCA (100 μM), LCA (50 μM) and DEX (50 μM) added as a 100-fold concentrated stock solution in ethanol (for $1,25(OH)_2D_3$), methanol (for CDCA and LCA) or DMSO (for DEX).

Rat liver slices were incubated in presence of both $1,25(OH)_2D_3$ (100 nM) and CDCA (50 μM) or PMA (1.6 μM); and with CDCA (50 μM) with PMA (1.6 μM). Furthermore, rat liver slices were incubated in presence of CDCA (50 μM) and $1,25(OH)_2D_3$ (100 nM) in the presence of the PKC inhibitor, Bis I (3 μM) for 2 h, 4 h and 8 h.

Rat liver slices were incubated for 8 h and 24 h, respectively. Human liver slices were incubated for 24 h. Control rat and human liver slices were incubated in supplemented Williams medium E with 1% ethanol, methanol, DMSO and ethanol or methanol + DMSO without ligands. From a single rat/single human liver donor, three replicate slices that were subjected to identical incubation conditions 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 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 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 intestine (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 (18).

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. CYP3A1 and CYP3A2 mRNA was analyzed by the SYBR Green detection system and the primer sequences used for villin, GAPDH, CYP3A1 and CYP3A2 analysis were identical to those reported earlier (18, 24). Rat VDR mRNA was analysed by SYBR Green and Taqman® analysis. VDR SYBR green primers are forward (5'-3') TGAAGGCTGCAAAGGTTTCT, and reverse primer (5'-3') TAGCTTGGGCCTCAGACTGT and VDR Taqman primers and probes are forward primer (5'-3') TGACCCCACCTACGCTGACT, reverse primer (5'-3') CCTTGGAGAATAGCTCCCTGTACT and probe (5'-3') FAM-ACTTCCGGCCTCCAGTTCGTATGGAC-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 (18). The human VDR primer sequence was: forward primer (5'-3') GGAAGTGCAGAGGAAGCGGGAGATG, reverse primer (5'-3') AGTGCTGGGACAGCTCTAGGGTCAC. 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 (18). 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, except for the rat and human VDR primer set which showed a minor second product. 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) (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 $2^{-(\Delta\Delta CT)}$.

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 VDR expression in rat intestine and liver. The VDR mRNA expression was found to be constant during control incubations of rat jejunum slices and slightly induced in ileum and colon slices (Fig. 1A) but was not influenced by the solvents during 8 h and 12 h of incubation (results not shown). Incubation of rat jejunum, ileum and colon slices with $1,25(OH)_2D_3$ for 12 h did not affect the VDR mRNA expression (Fig. 1B). Incubation of rat ileum slices with $1,25(OH)_2D_3$ for 8 h significantly induced VDR expression (1.7-fold; $P < 0.05$) (Fig. 2). DEX decreased the VDR mRNA expression in the ileum slices (0.6-fold; $P < 0.05$) but the small decrease in jejunum and colon slices was not significant (Fig. 1B). CDCA induced the VDR expression in the ileum slices during 8 h and 12 h incubation (2-2.5-fold; $P < 0.05$), (Figs. 1B and 2), but LCA decreased the VDR expression in the colon slices (0.5-fold; $P < 0.05$) (Fig. 1B). The synthetic FXR ligand, GW4064 and the PKCα agonist, PMA did not affect the VDR expression in the rat ileum slices (Fig. 2). Co-incubation of rat ileum slices with $1,25(OH)_2D_3$ and CDCA induced the VDR expression additively (3-fold; $P < 0.05$) (Fig. 2).

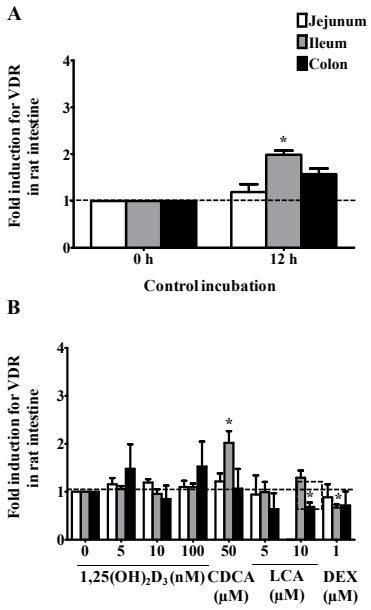

*Figure 1. Slices from rat jejunum, ileum and colon were exposed to control incubation (A), $1,25(OH)_2D_3$ (5-100 nM), LCA (5-10 μM), CDCA (50 μM) and DEX (1 μM) (B), for 12 h, after which total RNA was isolated and mRNA expression of VDR was evaluated by qRT-PCR. Results were expressed as fold-induction 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 2-3 rats; in each experiment 6 intestinal slices were incubated per condition. Significant differences towards the control incubations are indicated with * P < 0.05.*

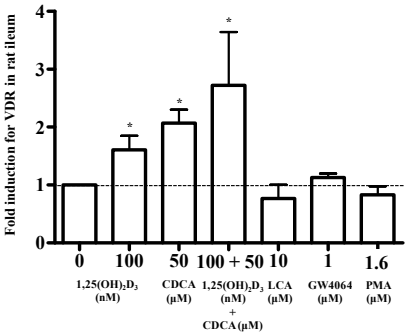

*Figure 2. Rat ileum slices were exposed to 1,25(OH)$_2$D$_3$ (100 nM), CDCA (50 μM), LCA (10 μM), GW4064 (1 μM) and PMA (1.6 μM) and co incubation with 1,25(OH)$_2$D$_3$ (100 nM) and CDCA (1-50 μM) (C) for 8 h, after which total RNA was isolated and mRNA expression of VDR was evaluated by qRT-PCR. Results 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 2-5 rats; in each experiment 6 ileum slices were incubated per condition. Significant differences towards the control incubations are indicated with * P < 0.05.*

In the rat liver slices, the VDR mRNA expression was significantly increased after 8 h and 24 h control incubation (Fig. 3A), but was not influenced by the solvents (results not shown). 1,25(OH)$_2$D$_3$, CDCA and PMA significantly induced VDR mRNA expression relative to their respective solvent incubated controls (Figs. 3B and C), whereas LCA and GW4064 had no effect. DEX significantly decreased the VDR mRNA expression in the liver slices (0.8-fold; $P < 0.05$) (Fig. 3B). Co-incubation of rat liver slices with 1,25(OH)$_2$D$_3$ and CDCA showed an additive induction of the VDR expression by 5.4-fold ($P < 0.05$) compared to incubation with the ligands individually (3.5-fold and 2.8-fold, respectively) (Fig. 3B). The PKCα inhibitor, Bis I abolished the induction of VDR mRNA by 1,25(OH)$_2$D$_3$, CDCA and PMA in rat liver slices after 2 h (Fig. 4) but not after 4 h and 8 h incubation (data not shown).

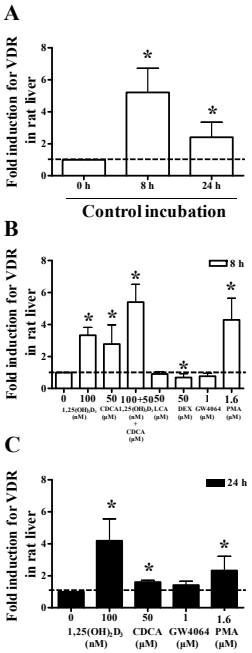

*Figure 3. Rat liver slices were exposed to control incubation; 0 h, 8 h and 24 h (A), 1,25(OH)$_2$D$_3$ (100 nM), CDCA (50 μM), LCA (50 μM), DEX (50 μM), GW4064 (1 μM) and PMA (1.6 μM) and 1,25(OH)$_2$D$_3$ (100 nM) + CDCA (50 μM) for 8 h (B), and 1,25(OH)$_2$D$_3$ (100 nM), CDCA (50 μM), GW4064 (1 μM) and PMA (1.6 μM) for 24 h (C), after which total RNA was isolated and mRNA expression of VDR 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 2-4 rats; in each experiment 3 liver slices were incubated per condition. Significant differences towards the control incubations are indicated with * P < 0.05.*

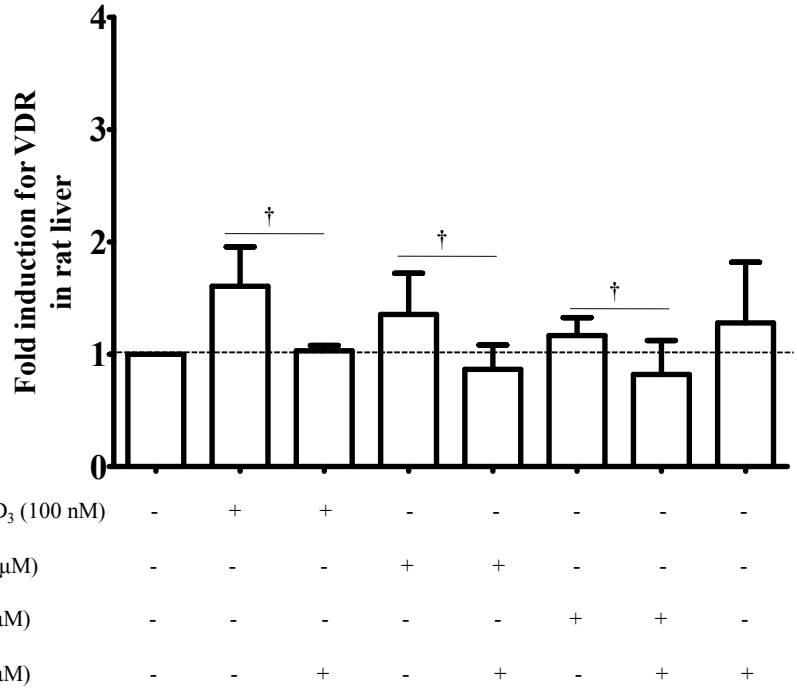

*Figure 4. Rat liver slices were exposed to control incubation, 2 h (A) and incubated with 1,25(OH)$_2$D$_3$ (100 nM), CDCA (50 μM) and PMA (1.6 μM) in the presence or absence of Bis I (3 μM) for 2 h, after which total RNA was isolated and mRNA expression of VDR was evaluated by qRT-PCR. Results were 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 rats; in each experiment 3 liver slices were incubated per condition. Significant differences towards the control incubations are indicated with * P < 0.05.*

Regulation of VDR in rat intestine, liver and kidneys – in vivo. The administration of 1,25(OH)$_2$D$_3$ to Wistar rats (1200 pmol/kg/day for 4 days by ip injection) did not affect the VDR mRNA expression in jejunum, ileum, colon and liver. The high variation in the liver data at 12 h was due to the finding that the liver of 1 out of 6 rats showed an induction, whereas no change was observed in the livers of other five rats. In contrast, the VDR mRNA expression was significantly induced in the kidneys after 4 days of treatment (2.5-fold induction; $P < 0.05$).

Regulation of CYP3A isozymes by the VDR ligands 1,25(OH)$_2$D$_3$ and LCA in the presence of CDCA in the rat ileum. The VDR ligands, 1,25(OH)$_2$D$_3$ and LCA, and the FXR ligand, CDCA significantly induced the expression of CYP3A1 (Fig. 6), and 1,25(OH)$_2$D$_3$ and LCA but not CDCA induced CYP3A2 expression in rat ileum after 8 h of incubation (Fig. 7). These results are consistent with our earlier results obtained after 12 h of incubation (18). CDCA strongly decreased the 1,25(OH)$_2$D$_3$ and LCA mediated

induction of CYP3A1 (Fig. 6) and the $1,25(OH)_2D_3$ mediated CYP3A2 induction in a dose-dependent manner (Fig. 7) in rat ileum slices. CDCA did affect the LCA mediated CYP3A2 induction.

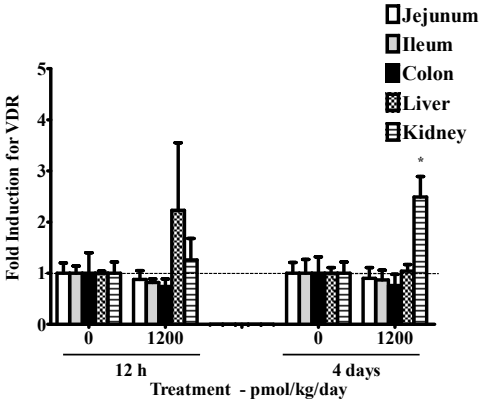

*Figure 5. Wistar rat were treated with with $1,25(OH)_2D_3$ (1200 pmol/kg/day) for 12 h and 4 days by intraperitoneal injection. After 12 h or 24 h of the last dose administered, rats were sacrificed and total RNA was isolated from jejunum, ileum, colon, liver and kidneys and mRNA expression of VDR was evaluated by qRT-PCR. 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 6 rats.. Significant differences towards the vehicle treated rats were indicated with * P < 0.05.*

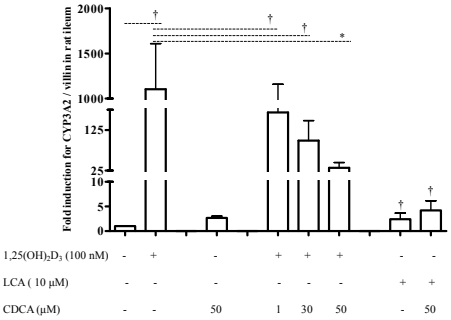

*Figure 6. Rat ileum slices were exposed to $1,25(OH)_2D_3$ (100 nM), CDCA (50 μM) and LCA (10 μM), co incubated with $1,25(OH)_2D_3$ (100 nM) and CDCA (1-50 μM) and co incubated with LCA (10 μM) and CDCA (50 μM) for 8 h, after which total RNA was isolated and mRNA expression of CYP3A1 was evaluated 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. Results showed mean ± S.E.M. of 3-4 rats; in each experiment 3-6 ileum slices were incubated per condition. Significant differences towards the control incubations are indicated with * P < 0.05. "†" denotes in all the experiments but failed to reach statistical significance.*

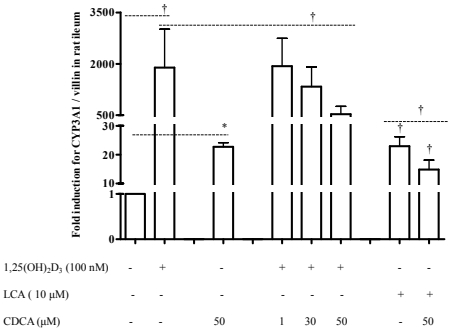

*Figure 7. Rat ileum slices were exposed to $1,25(OH)_2D_3$ (100 nM), CDCA (50 μM) and LCA (10 μM), co incubated with $1,25(OH)_2D_3$ (100 nM) and CDCA (1-50 μM) and co incubated with LCA (10 μM) and CDCA (50 μM) for 8 h, after which total RNA was isolated and mRNA expression of CYP3A2 was evaluated 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. Results showed mean ± S.E.M. of 3-4 rats; in each experiment 3-6 ileum slices were incubated per condition. Significant differences towards the control incubations are indicated with * P < 0.05. "†" denotes decrease in all the experiments but failed to reach statistical significance.*

Regulation of VDR expression in human ileum and liver. The VDR mRNA was clearly detected in the human ileum. In the human ileum slices, the VDR mRNA expression was found to be slightly decreased during 8 h and 24 h control incubation, but was not influenced by the organic solvents (data not shown). Incubation of human ileum slices with $1,25(OH)_2D_3$ induced the VDR expression after 24 h (Fig. 8A). LCA, CDCA and DEX did not affect the VDR expression (Fig. 8A) but PMA induced the VDR expression in the human ileum slices (Fig. 8A). In the human livers, the VDR expression showed high variability and was detectable at a C_T of 33 to 38 whereas in one liver VDR expression could not be detected up to 40 cycles. In the human liver slices, the VDR expression was slightly increased during 24 h incubation but was not influenced by the organic solvents (data not shown). $1,25(OH)_2D_3$ did not affect the VDR expression, whereas CDCA, LCA and DEX decreased the VDR mRNA expression in all the human livers (Fig. 8B).

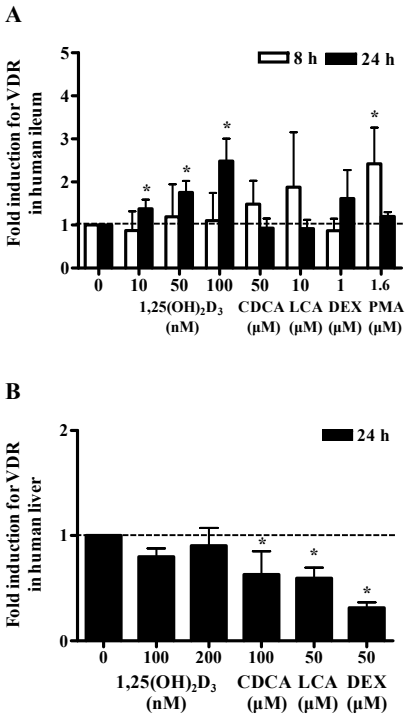

*Figure 8. Human ileum slices were exposed to $1,25(OH)_2D_3$ (10-100 nM), CDCA (50 µM), LCA (10 µM), DEX (1 µM) and PMA (1.6 µM) for 8 h and 24 h (A), and human liver slices were exposed to $1,25(OH)_2D_3$ (100-200 nM), CDCA (100 µM), LCA (50 µM) and DEX (50 µM) for 24 h (B), after which total RNA was isolated and mRNA expression of VDR was evaluated by qRT-PCR. Results were expressed as fold-induction after normalizing with villin (for ileum) or GAPDH (for liver), 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 and liver donors; in each experiment 3 ileum and liver slices were incubated per condition. Significant differences towards the control incubations are indicated with * $P < 0.05$.*

Discussion

VDR mRNA is expressed along the length of the rat intestine as well as in the liver. In the rat intestine, VDR protein was found to be localized by immunohistochemistry in the epithelial cells, whereas in the rat liver, VDR was localized in the non-parenchymal cells with predominant localization in the cholangiocytes. In the human liver VDR is expressed in both the cholangiocytes and to a lesser extent in the hepatocytes. In the human ileum, the VDR expression is localized in the epithelial cell (Chapter 4).

We report here for the first time the induction of VDR mRNA in the rat and human ileum by $1,25(OH)_2D_3$. Incubation of rat ileum slices with $1,25(OH)_2D_3$ for 8 h significantly induced the VDR expression by 1.7-fold; $P < 0.05$ (Fig. 2), but this effect appeared to be transient, since at 12 h, induction of VDR was not found (Fig. 1B). However, *in vivo* VDR induction was not found after 12 h and 4 days of treatment in any of the regions of the intestine (Fig. 5). Recently, Chow et al. found an induction of VDR in the jejunum but not in the ileum of Sprague-Dawley rats treated with a high dose of $1,25(OH)_2D_3$ (2560 pmol/kg/day) for four days (2). In the human ileum slices, similar to the rat ileum, $1,25(OH)_2D_3$ induced the VDR expression (Fig. 8A). To the best of our knowledge this is the first data on VDR induction by $1,25(OH)_2D_3$ in man. Furthermore, $1,25(OH)_2D_3$ induced the VDR expression in rat liver slices (Fig. 3B). However, treatment of Wistar rats with $1,25(OH)_2D_3$ (1200 pmol/kg/day) either for 12 h or 4 days did not induce the VDR expression in the rat liver (Fig. 5) but Chow et al. found induction of VDR in the livers of Sprague-Dawley rats at high dose of $1,25(OH)_2D_3$ (2560 pmol/kg/day) (un published observation). The induction of VDR expression observed in the kidneys (Fig 5) was also found by Wiese et al. (46), Healy (2005) and Chow et al (2) and showed that the dose was high enough to give a response in the kidney.

To investigate whether the concomitant increase in bile salt concentration in the portal blood due the increased absorption of bile salts in the rat ileum by the $1,25(OH)_2D_3$ mediated induction of ASBT (2) could explain the absence of an effect *in vivo* in the liver, we co-incubated liver slices with $1,25(OH)_2D_3$ and CDCA. We found an additive effect on the VDR induction *in vitro* (Fig. 3B). Surprisingly, LCA, a high affinity ligand for VDR, and a low affinity ligand for PXR and FXR did not induce VDR expression in rat ileum and liver slices (Figs. 1B, 2 and 3B). In contrast, CDCA, which is a high affinity ligand for FXR and a low affinity ligand for VDR, induced the VDR expression in the rat ileum and liver slices (Figs. 1B, 2 and 3B). To the best of our knowledge, this effect of CDCA was not reported before. The lack of induction of VDR by LCA, and the finding that the CDCA, which exhibits moderate affinity to VDR compared to LCA induced VDR in ileum and liver slices (Figs. 2 and 3B). These results suggest that CDCA and $1,25(OH)_2D_3$ mediate the VDR expression by a VDR-independent pathway. This is further supported by the finding that both $1,25(OH)_2D_3$ and LCA showed an induction of the expression of the well known VDR target genes, CYP3A1 and CYP3A2 (Figs. 6 and 7), which is consistent with our earlier findings (18).

Species differences were observed for the regulation of VDR expression by LCA and CDCA. In contrast to rat ileum and liver, where CDCA increased the VDR expression, CDCA did not affect the VDR expression in human ileum slices (Fig. 8A) and decreased VDR expression in human liver slices (Fig. 8B). LCA did not affect the VDR expression in the human ileum, similar to the rat ileum, but decreased the VDR expression in the human liver, in contrast to the lack of effect in the rat liver. These observations emphasize the species and organ specific regulation of VDR. This cannot be explained by a lack of uptake

of the ligands into the cells of the ileum or liver as we have previously found up regulation of SHP expression by CDCA, and up regulation of OSTα and OSTβ by LCA and CDCA in human ileum and human liver slices (17).

To further gain insight in the nuclear receptor pathways involved in the $1,25(OH)_2D_3$ and CDCA mediated VDR induction in the rat ileum and liver slices, we incubated the rat ileum and liver slices with the synthetic FXR ligand, GW4064 and the PKCα ligand, PMA, as CDCA is known to interact with FXR, but also activates PKCα. Furthermore, PKCα is reported to up regulate VDR expression (20, 22, 34, 37). The FXR ligand, GW4064 did not affect the VDR expression in rat ileum and liver slices (Figs. 2, 3B and 3C), but induced the short heterodimer protein (SHP) (A.A. Khan et al., un published observation), as expected for FXR ligands (9), suggesting that the FXR pathway is intact in the slices and that the FXR is not involved in the regulation of VDR. In contrast, PMA induced the VDR expression in the rat liver slices, confirming the role of PKCα in the regulation of VDR expression in rat liver slices. Furthermore, the induction of VDR by CDCA, PMA and $1,25(OH)_2D_3$ was inhibited by the PKCα inhibitor, Bis I during 2 h of incubation (Fig. 4), which indicates that the effect of CDCA and $1,25(OH)_2D_3$ in the liver might be mediated via the PKCα pathway. Bis I mediated inhibition of VDR induction by CDCA and $1,25(OH)_2D_3$ in rat liver slices was not observed for 4 h and 8 h incubation. This might be probably related to the rapid metabolism of Bis I. Also in the human ileum, PKCα seems to be involved in the regulation of VDR expression (Fig. 8A). The lack of effect of PMA on the expression of VDR in the rat ileum is difficult to explain with the current data, but the PMA effect was tested at one incubation time point only. Further studies are needed to confirm the role of PKCα in the human liver and rat ileum. The GR ligand DEX decreased the VDR expression in rat ileum, rat liver and human liver slices (Figs. 1B, 3B and 8B), but not in human ileum slices (Fig. 8A). Our results show for the first time that the expression of VDR in rat ileum and liver and human liver is decreased in the presence of glucocorticoids. A repression of VDR by glucocorticoids was reported before for the mouse intestine (13), but not for the liver, and is in contrast to the earlier reports in rat intestine in vivo (14)

Further, we investigated the effect of the CDCA mediated induction of VDR in rat ileum on the regulation of CYP3A isozymes: CYP3A1 and CYP3A2, since these enzymes are reported to be induced by VDR ligands, $1,25(OH)_2D_3$ (18) and LCA (Chapter 3) in rat ileum slices. Hence, a synergistic increase in the induction of CYP3A1 and CYP3A2 was expected for $1,25(OH)_2D_3$ and LCA in rat ileum slices in the presence of CDCA due to the induction of VDR. Such an additive effect was reported earlier for $1,25(OH)_2D_3$ mediated induction of CYP24A1 in rat osteosarcoma cells after pretreatment with PTH for 4 h, (19). In contrast to the expected increase in CYP3A1 and CYP3A2 induction, CDCA strongly decreased the $1,25(OH)_2D_3$ mediated induction of CYP3A1 and CYP3A2 in a dose dependent manner (Figs. 6 and 7) and also decreased the LCA mediated induction of CYP3A1 (Figs. 6 and 7) but not that of CYP3A2 (Fig. 7). The CDCA mediated repression

of CYP3A induction by VDR ligands in rat ileum suggest that the toxicity of LCA might be potentiated in the presence of CDCA by inhibiting its metabolism, which is predominantly mediated by feed forward induction of CYP3A1 and CYP3A2 in rat intestine and CYP3A4 in human ileum by VDR (18) and (Chapter 3). Whether this interaction also occurs in human ileum is currently under investigation. This might be of importance in people consuming high fat diet, which is associated with an increase in the faecal excretion of bile acids (36), including LCA and CDCA. LCA is implicated as a carcinogenic agent in the intestine and as a cholestatic agent in the liver of rats and man (7, 15, 21, 30), because it forms DNA adducts and DNA strand breaks and inhibits the DNA repair enzyme, DNA polymerase β (31). Thus, CDCA might potentiate the LCA mediated colon carcinogenesis by repressing the VDR-liganded induction of CYP3A isozymes by LCA. This might be one of the possible mechanisms of increased incidence of colon cancer in populations consuming high fat diet.

In conclusion, we have studied the regulation of VDR mRNA expression in rat and human ileum and liver and found that $1,25(OH)_2D_3$, DEX, CDCA and LCA are involved in its regulation. Moreover, we found prominent species and organ differences in the regulation of VDR by bile acids and $1,25(OH)_2D_3$. The regulation of VDR by $1,25(OH)_2D_3$ and CDCA is not likely to be mediated by VDR or FXR activation but we found indications that in rat liver and human ileum, PKCα seems to be involved, which could not be confirmed for the rat ileum and human liver. In contrast to what was observed in the rat liver, CDCA and LCA decreased the VDR expression in human livers. Further, glucocorticoids decreased the VDR expression in rat and human liver and also in rat ileum but not in human ileum, which indicates a cross talk between VDR and GR. In addition, we identified a possible novel mechanism of enhancement of LCA mediated toxicity in ileum by potential inhibition of VDR mediated induction of CYP3A isozymes by the simultaneous presence of CDCA and LCA in the rat ileum. Further studies are in progress to investigate whether a similar mechanism plays a role in the regulation of CYP3A4 in human ileum.

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Chapter 8
General Discussion and Conclusions

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1 Introduction

The research described in this thesis is focused on the regulation of drug metabolizing enzymes (DMEs) and drug transporters (DTs) that are involved in drug metabolism and transport as well as in bile acid synthesis, detoxification and transport in human and rat liver and intestine. To predict drug metabolism and transport of drugs in man, animal studies are widely applied and among the rodents, the rat is the most preferred preclinical animal species used in the pharmacological and toxicological evaluation of new chemical entities (NCE) in drug discovery. However interspecies differences make this extrapolation hazardous and unreliable. Moreover the expression of DMEs and DTs is subject to regulation by ligand-activated nuclear receptors (NRs) and nuclear factors (NFs). Data on regulation of DMEs and DTs in animals can be obtained from *in vivo* studies and data on regulation of DMEs and DTs in man is hard to obtain. For human studies occasionally tissue from patients is studied but it remains undetermined to what extent confounding factors of the disease interfere with the results, and moreover secondary effects of ligands cannot be discriminated from primary effects. Therefore *in vitro* methods are required to study the direct effects of drugs on the regulation of DMEs and DTs. *In vitro* methods to study regulation of DMEs and DTs in liver are available, such as primary human hepatocytes, immortalized cell lines and precision cut liver slices (PCLS) but for the intestine very few successful methods were available, such as enterocytes and intestinal derived immortalized cell lines (Caco-2) and data on regulation in the intestine are scarce. Recently, we validated precision cut intestinal slices (PCIS) as an *in vitro* model to study activity and regulation of DMEs. In this model all the cell types are represented and the cell-matrix contacts remain intact. They can be prepared from human liver and intestine in a similar way and allow studying the regional differences in the activity and regulation of DMEs and DTs in the intestine. Therefore we set out to study the regulation of DMEs and DTs in human and rat tissue slices, prepared from liver and from different regions of the intestine.

In this chapter, the results on induction and repression of DMEs, DTs and the NRs / NFs involved in the regulation of DMEs and DTs by ligands of the pregnane X receptor (PXR; NR1I2), glucocorticoid receptor (GR; NR3C1), farnesoid X receptor (FXR; NR1H4) and vitamin D receptor (VDR; NR1I1) in human intestine and liver are summarized and discussed. Furthermore, the data obtained in human tissue are compared to those in rat tissue and the observed species differences are discussed.

The most preferred route of administration of drugs is the oral route. Although, the liver is the most important organ for the first pass metabolism of orally ingested drugs, nowadays the intestine is also recognized as a major drug metabolism organ with the identification of high levels of DMEs and DTs, and clinical drug interactions are reported at the level of intestinal metabolism for drugs such as cyclosporine, midazolam and verapamil based on pharmacokinetic studies in man (27, 42, 72). During drug discovery, it is

important to screen the NCEs for its intestinal drug metabolic profile along with the liver metabolic profile. After oral administration, the enterocytes are exposed to very high concentrations of the ingested drugs in the intestinal lumen and they can have a significant impact on the bioavailability of these drugs. Induction and repression of DMEs and DTs in response to environmental stimuli and co-administered drugs may influence the oral bioavailability of drugs. This needs to be investigated during drug discovery and development and not only in the preclinical animal species but also in humans. This is particularly of importance for drugs which have a narrow therapeutic window because of the imminent danger of decreased efficacy or adverse drug reactions, such as described for immunosuppressive agents like cyclosporin or tacrolimus. These drugs are substrates for CYP3A4 and the efflux pump, multi drug resistance protein 1 (MDR1 or Pgp). Induction and repression of CYP3A4 and MDR1 by co-administered drugs can have a profound impact on the bioavailability, efficacy and toxicity of these compounds (27, 42, 72). Hence, understanding the underlying mechanisms for the induction and repression of DMEs and DTs in the intestine and the liver is of paramount importance. Many mechanisms are known to be involved in the regulation of the expression of DMEs and DTs, and constitutive, induced and repressed expression of genes is largely controlled at the level of transcription by the involvement of nuclear receptors, such as the PXR, constitutive androstane/active receptor (CAR), GR, VDR, aryl hydrocarbon receptor (Ahr) and Nrf2 (40, 41, 48, 51, 57, 70). **In this thesis, we aimed to investigate the VDR mediated induction and repression of DMEs and DTs involved in drug and bile acid detoxification, transport and synthesis proteins in human and rat liver and intestine using precision-cut tissue slices as *in vitro* model.** To obtain more insight in the role of VDR and to elucidate the role of other nuclear receptors in bile acid synthesis and disposition, the effects of the VDR ligands, 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and lithocholic acid (LCA) were compared with those of specific ligands for various other nuclear receptors, chenodeoxycholic acid (CDCA) and GW4064 for FXR, pregnenolone-16α carbonitrile (PCN) for PXR, budesonide (BUD) for glucorticoid receptor (GR) and dexamethasone (DEX) for GR and PXR.

In vitro studies like reporter gene assays are powerful tools to characterize the nuclear receptor response elements (NRRE's) in the promoters of the DMEs and DTs. Primary cultures of rat and human hepatocytes and enterocytes, and immortalized human cell lines such as HepG2, LS180 and Caco-2 are widely applied to study the effect of various ligands on DMEs and DTs (15, 34, 35, 43, 54, 56). However, the induction and repression of DMEs and DTs in the intestine and liver in response to ligands of the NRs is not only determined by the presence of NRRE's in the target genes but also by the expression levels of the NRs, co-activators and repressors in the tissues, which in their turn are also subjected to regulation and are not always expressed at their original levels in these cell lines. Moreover, the exposure of the particular cell to the ligand plays an important role. This exposure is determined by the extracellular concentration and by the rate of uptake, metabolism and excretion of the ligand. This may vary between the different

organs, and hence the response may vary concomitantly. Therefore in **chapter 2 and 4**, we characterized the expression of the nuclear receptors, VDR, PXR and FXR at the level of mRNA along the length of the rat intestine and found significant differences in different regions of the intestine, which we further confirmed at the level of protein by immunohistochemistry for VDR. VDR mRNA was found to be highly expressed in the enterocytes of the rat intestine with an increasing gradient from jejunum to colon, and also in human ileum and colon. In the rat and the human liver, the VDR mRNA expression was found to be very low but detectable. The cellular localization of the VDR protein in the liver showed a species difference: in the rat liver VDR is localized exclusively in the bile duct epithelial cells and endothelial cells, whereas in the human liver, VDR protein could also be detected in the hepatocytes along with the bile duct epithelial cells and the endothelial cells. This gradient in expression is not found for every NR. The expression of PXR and FXR also showed a gradient along the length of the intestine with the highest expression in colon, but their expression was 2-10 fold higher in the liver compared to the intestine. However taking into account that the enterocytes represent ~ 17-25% of the intestinal tissue and the hepatocytes represent ~ 80% of the liver tissue, the expression of PXR and FXR in enterocytes is rather similar to that in hepatocytes. Previous data from our laboratory showed that the CAR expression did not vary much along the length of the rat intestine (**van de Kerkhof et al., thesis 2007, unpublished observation)**. Future research should elucidate the relative expression of these NRs in human jejunum, ileum, colon and liver. Currently the expression of the NRs is further investigated at the protein level by immunohistochemistry.

2 Regulation of CYP3A isozymes by VDR, PXR, GR and FXR in rat and human intestine and liver

The cytochrome P450 (CYP P450) 3A isozymes are the most abundant CYP isozymes expressed in the liver and the intestine, and are responsible for the metabolism of 60% of the marketed drugs and a number of endogenous compounds such as bile acids (18, 23, 50). In **chapter 2**, we studied the role of the four nuclear receptors in the regulation of CYP3A isozymes at the level of mRNA in different regions of the rat intestine and liver, and the human ileum and liver with VDR-, PXR-, GR- and FXR-specific ligands: $1,25(OH)_2D_3$, LCA, PCN, DEX, BUD and CDCA using precision-cut tissue slices. In the rat intestine, CYP3A1 expression is very sensitive to the VDR ligand, and to a lesser extent to PXR and GR ligands and the induction showed a gradient in the different segments of the intestine which was different for the different ligands but did not correlate with the gradient of the respective NRs. For the VDR ligand, the gradient was in the rank order of ileum (Il) > jejunum (J) = colon (Co). The CYP3A2 expression was found to be exclusively regulated by the VDR ligand and only in the ileum. The CYP3A9 expression in the liver and in all regions of the intestine appears to be mainly regulated by PXR and GR ligands but not by the VDR ligand. In rat liver slices, $1,25(OH)_2D_3$ did not alter the expression of CYP3A isozymes, which can be explained by the specific localization of

VDR in the bile duct epithelial cells and the very high expression of CYP3A isozymes in the hepatocytes. A potential up regulation of CYP3A isozymes in bile duct epithelial cells is therefore difficult to detect. The $1,25(OH)_2D_3$ mediated induction of CYP3A1 mRNA in rat intestinal slices, presumably mediated through VDR is confirmed in *in vivo* studies in Wistar rats treated with 1200 pmol/kg/day $1,25(OH)_2D_3$ intraperitoneally **(chapter 5)**, which is in line with the earlier reports of Xu et al.(73) and Chow et al. in Sprague-Dawley rats treated with $1,25(OH)_2D_3$ intraperitoneally (640 – 2560 pmol/kg/day) (14). However, induction of CYP3A1 mRNA *in vivo* was found to be high in the jejunum compared to the ileum, where it showed a large variation. Furthermore, CYP3A1 mRNA was not induced in the colon of these rats and CYP3A2 mRNA was not induced in the ileum. These results are in line with the results of Chow et al. (14). The apparent contrasting results of the *in vivo* studies to those of the slice experiments, where, $1,25(OH)_2D_3$ showed a significant induction of CYP3A1 mRNA along the length of the intestine and CYP3A2 in ileum **(chapter 2 and 3),** might be due to the concentration gradients in the intestinal blood, resulting in a different exposure of the enterocytes along the length of the intestine.

Further, in **chapter 2** we studied the $1,25(OH)_2D_3$-mediated regulation of CYP3A4 in human ileum and liver. $1,25(OH)_2D_3$ significantly induced the CYP3A4 expression in human ileum and liver slices, similar to earlier studies in Caco-2, LS180 HepG2 cells and human hepatocyte cultures (20, 24, 49, 62, 63). However, $1,25(OH)_2D_3$ mediated induction of CYP3A4 in human liver shows a high variation among the human liver donors which can be attributed to the variation in the VDR expression. Furthermore, both in the ileum and the liver, CYP3A4 was up regulated by VDR, PXR and GR ligands.

Our results suggest that prediction of the inducing potential of drugs based on reporter gene assays should not rely strictly on whether or not the drug under study is a ligand for a certain NR, or whether the promoter of the target gene harbors the NRRE. Also the expression level of the NR in the target organ is not predictive for the inducing potential. The uptake, metabolism and excretion of the ligand as well as the availability of co-activators or repressors in the specific tissue may play a decisive role in the regulation of DMEs and DTs. For example, the VDR ligand, $1,25(OH)_2D_3$, is likely to be highly metabolized in the colon compared to the jejunum due to the higher expression of CYP24A1 in rat colon compared to jejunum **(Chow et al., un published observation)**, which may explain its relatively low effect of $,25(OH)_2D_3$ in the colon despite the high VDR expression. All these factors may show interspecies and inter organ differences.

In **chapter 3**, we investigated the effects of another VDR ligand, LCA (47), a toxic bile acid formed by the bacterial biotransformation of the primary bile acid CDCA in the terminal part of the ileum (16, 30) on the regulation of CYP3A isozymes in the rat intestine and liver, and the human ileum and liver at the level of mRNA. LCA, similar to $1,25(OH)_2D_3$ (**chapter 2**) induced the expression of CYP3A1 along the length of the intestine with the highest induction in ileum slices, and CY3A2 in ileum slices only.

Furthermore, in contrast to $1,25(OH)_2D_3$, LCA also induced the expression of CYP3A9 in liver slices and along the length of the intestine with a moderately higher induction in the colon slices. This effect is presumably mediated through PXR, as $1,25(OH)_2D_3$ did not regulate this isoform and can be explained by the observation that LCA and its metabolite, 3-keto-5β-cholanic acid (3KCA) also bind to PXR (66). The PXR mediated induction of the CYP3A9 isozyme in the rat intestine and liver was further confirmed by the effects of the synthetic PXR ligands, PCN and DEX in rat intestine and liver slices **(chapter 2 and 3)**. Unexpectedly, LCA did not induce CYP3A1 expression in rat liver slices, although CYP3A1 is regulated by PXR ligands such as PCN and DEX in rat intestine and liver slices **(chapter 2 and 3)** but an induction of CYP3A9 was found, presumably via PXR. Further experiments are needed to explain this discrepancy. In addition, we found indications for a role for GR in the regulation of the rat CYP3A9, as it was also up regulated by the GR ligand, BUD in the rat intestine and liver **(chapter 2 and 3)**, which to the best of our knowledge has not been reported before and deserves further investigations. Furthermore, in human ileum slices, LCA similar to $1,25(OH)_2D_3$ induced CYP3A4 **(chapter 2 and 3)**. However, surprisingly, the induction of CYP3A4 by LCA in human liver slices did not correlate with the expression of VDR and LCA even decreased the expression of CYP3A4 in three out of four VDR positive livers. These results suggest that the induction of CYP3A4 by LCA in human livers is mediated through other nuclear receptors, such as the PXR, which in man is referred as steroid X receptor / pregnane activated receptor (SXR/PAR) and FXR, since DEX and CDCA significantly induced CYP3A4 in human liver slices, which is consistent with the earlier reports in human hepatocytes (28). The effect of VDR, FXR, SXR/PAR and GR ligands on CYP3A4 regulation in individual human livers is summarized in Tables 1, 2, 3 and 4. The results obtained with the various nuclear receptor ligands in human liver and ileum are highly variable with respect to extent of the regulation, but are qualitatively consistent with previously published data, showing the validity of the model. In spite of that, the LCA effects did not seem to correspond to the known effects of VDR, PXR and FXR ligands **(chapter 3)** (Tables 1, 2, 3 and 4). Toxicity of LCA in the human liver slices at these doses cannot be excluded, although the significant up regulation of the mRNA of OSTα and OSTβ seems in contradiction with toxicity **(chapter 6)**. Further experiments are needed to elucidate the mechanism of the effects of LCA in the human liver.

In **chapter 7**, we investigated the interaction of CDCA, with, $1,25(OH)_2D_3$ and LCA, on the regulation of CYP3A isozymes involved in LCA detoxification. CDCA is known to induce the expression of VDR but in spite of inducing VDR **(chapter 7)** it decreased the induction of CYP3A1 and CYP3A2 in the presence of known VDR ligands, $1,25(OH)_2D_3$ and LCA. These enzymes are involved in BA detoxification. Hence, our results suggest a possible novel mechanism of enhancement of LCA-mediated toxicity in ileum by inhibiting the VDR-mediated induction of CYP3A isozymes by the simultaneous presence of CDCA and LCA in the rat ileum. Further studies are in progress to investigate whether a similar mechanism plays a role in the regulation of CYP3A4 in the human ileum.

3 Regulation of bile acid transporters by VDR, PXR, GR and FXR in rat and human intestine and liver.

In **chapter 4, 5 and 6**, we studied the regulation of the BA transporters as well as the NRs involved in the regulation of these proteins in rat and human ileum and liver by VDR, PXR, GR and FXR ligands. BAs are synthesized from cholesterol (60) in the liver by a cascade of 12 reactions, initiated by the first and rate limiting step, cholesterol 7α-hydroxylation, catalyzed by CYP7A1 (13) to form CDCA and cholic acid (CA), which upon amidation are transported into the bile canaliculi by the bile salt export pump (BSEP) and secreted into the intestine via the bile. About 95 % of the bile acids are actively re-absorbed in the terminal ileum by the coordinate action of apical sodium dependent bile acid transporter (ASBT; SLC10A2) and the basolateral heterodimeric organic solute transporter (OSTα-OSTβ) (5, 65). In the liver, bile acids are taken up into hepatocytes by the sodium dependent co transporting polypeptide (NTCP; SLC10A1) and members of the organic anion transporting polypeptide (OATP) family. Bile acid synthesis and secretion in the liver, reabsorption in the ileum and uptake in the liver constitute the enterohepatic cycle (33), which maintains the bile acid pool *in vivo* and is coordinately modulated by regulation of the proteins involved in the synthesis, transport and reabsorption of BAs. BAs play an important role in the absorption of lipids and lipid soluble vitamins in the upper part of the intestine.

The reabsorption of BAs in the terminal part of the ileum is mediated by the ASBT, which is highly regulated at the level of transcription by various nuclear receptors. The peroxisome proliferator activated receptor α (PPARα; NR1C1) and the GR were reported to mediate the expression of ASBT in rat and human ileum (37, 38, 53). Recently, Chen et al. (11) reported the involvement of the VDR in the regulation of ASBT in the rat ileum, but data on the involvement of VDR in the expression of ASBT in the human ileum is lacking up to now. Furthermore, in mice, guinea pigs, and rabbits ASBT was reported to be negatively regulated in the ileum by the reabsorbed BAs mediated through the FXR (45, 46, 52, 71). The ligand activated GR, PPARα and VDR bind to their respective response elements in the ASBT promoter as a homodimer or heterodimer with retinoic acid X receptor α (RXRα; NR1B1) (38, 69) and induce the ASBT expression. The BA-activated FXR forms a heterodimer with RXRα and induces the expression of short heterodimer protein (SHP; NR0B2), which down regulates the ASBT expression by inhibiting the activity of liver receptor homologue–1 (LRH-1; NR5A2), which is essential for the basal expression of ASBT. In the rat, the regulation of the ASBT gene by BAs is controversial with reports suggesting positive regulation (29, 67), negative regulation (21, 61) or no regulatory effects (3). Recently, 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 a negative feedback regulation of ASBT by the BA-liganded FXR in the rat, as the FXR-SHP-LRH1 cascade cannot play a role in the rat (10).

In chapter 5, we characterized the VDR, PXR, GR and FXR mediated regulation of ASBT and the NRs regulating its expression in rat and human ileum and liver precision-cut tissue slices. Consistent with earlier reports (37, 53), the GR ligand DEX significantly induced the expression of ASBT in rat and human ileum and liver slices, suggesting an intact and responsive GR pathway. The VDR ligand, $1,25(OH)_2D_3$ significantly decreased the expression of ASBT mRNA in rat ileum and liver slices, and in the human ileum but not in the human liver. The $1,25(OH)_2D_3$ mediated ASBT repression in rat ileum slices is in contrast to the data of Chen et al.(11), who showed a positive VDRE in rat ASBT promoter and induction of ASBT mRNA expression and activity in the ileum of Sprague-Dawley rats with high doses of $1,25(OH)_2D_3$. We speculate that this difference might be due to a difference in sensitivity between strains of rats as treatment of Wistar rats *in vivo* with $1,25(OH)_2D_3$ did not affect the ASBT expression in ileum **(chapter 5)**. Moreover, more recent experiments in Sprague-Dawley rats could not confirm this mRNA induction, but showed an increased protein expression at the highest dose only (14). The differences observed in the regulation of ASBT between the *in vitro* (ileum slices) and *in vivo* experiments in Wistar rats might be ascribed to a lower exposure of ileocytes to the effective concentration of $1,25(OH)_2D_3$ in ileum *in vivo* after the intraperitoneal administration, as we also found a much lower and highly variable CYP3A1 induction *in vivo* as compared to the high and consistent induction *in vitro* in ileum slices cultured in the presence of $1,25(OH)_2D_3$ **(Chapters 2 and 5).** We did not measure the plasma concentration of $1,25(OH)_2D_3$, but we can estimate, based on the data of Brown et. al. (9) who measured serum levels after ip injection of radiolabelled $1,25(OH)_2D_3$ that the serum levels in our rats are 3-5 nM and are lower than those in the *in vitro* incubations.

Interestingly, we found a repression of HNF1α in rat ileum slices treated with $1,25(OH)_2D_3$ **(Chapter 5)**, since HNF1α is essential for the basal expression of ASBT (64). The repression of ASBT may be explained by this repression of HNF1α. These results were further confirmed with the natural VDR ligand LCA, which also showed a significant repression of ASBT in rat ileum with concomitant repression of HNF1α. Like in rat ileum slices, $1,25(OH)_2D_3$ decreased the ASBT expression in rat liver slices, in line with the co-localization of VDR and ASBT in the biliary epithelial cells. $1,25(OH)_2D_3$ also decreased the ASBT expression in the human ileum slices, whereas in the human liver slices ASBT expression remained unaltered, which is as yet unexplained but indicates species and organ specific differences in the regulation of ASBT in rat and human ileum and liver.

The FXR ligands, CDCA and GW4064 did not affect the ASBT expression in rat ileum slices, indicating that FXR does not play a significant role in the ASBT regulation in the rat ileum in spite of an intact FXR pathway as shown by the SHP induction in these samples. This can be attributed to the reported lack of LRH1 binding site in the rat ASBT promoter (10), which is essential for the feedback regulation by FXR through the FXR-SHP-LRH-1 cascade (52). However, CDCA induced the ASBT expression in rat liver slices, which indicates an LRH-1-independent pathway in the biliary epithelial cells. In line

with the results published by Bergheim et al. (7), CDCA significantly decreased the ASBT expression in human ileum slices with concomitant induction of SHP (7), but did not affect the ASBT expression in the human liver, which may indicate a lower expression of FXR in the biliary epithelial cells than in the enterocytes.

Subsequent to characterizing the regulation of ASBT in rat and human ileum and liver by VDR, FXR, GR and PXR nuclear receptor ligands, we studied the regulation of basolateral and canalicular bile acid transporters involved in the excretion of bile acids in the rat and human ileum and liver in **chapter 6**. Several basolateral bile acid transporters such as truncated ASBT (tASBT), MRP3 and MRP4, which shows an affinity towards BAs have been proposed to be involved in bile acid excretion (35, 44, 59, 68). However, the organic solute transporter (OST) consisting of two half transporters, α and β (OSTα and OSTβ) is recently reported as the main bile acid transporter and its expression on the basolateral membranes parallels the ASBT expression along the length of the intestine, bile duct epithelial cells and renal proximal tubular cells in mouse, rat and human (4, 17). Furthermore Ostα knockout mouse showed perturbed bile acid homeostasis (6, 58). The regulation of OSTα and OSTβ is largely unknown and therefore we studied the regulation of OSTα and OSTβ in the rat and human intestine and the liver. Incubation of rat and human ileum and liver slices with the FXR ligand, CDCA showed the induction of the OSTα and OSTβ genes, confirming earlier observations (43). Furthermore, our experiments showed for the first time that CDCA also induced the OSTα and OSTβ gene expression in rat jejunum and colon. The rat but not the human OSTα and OSTβ genes in the ileum were negatively regulated by the VDR ligands, $1,25(OH)_2D_3$ and LCA. This repression of rat OSTα and OSTβ genes by $1,25(OH)_2D_3$ led to the postulation of a negative VDRE in the rat promoters, which remains to be confirmed. OSTα and OSTβ are positively regulated by the GR ligand, DEX but not by the PXR ligand. In conclusion, rat and human OSTα and OSTβ genes are positively regulated by FXR and GR ligands., and, in the rat the VDR ligand decreases the expression of both apical and basolateral bile acid transporters, ASBT, OSTα and OSTβ . Further studies are needed to evaluate the role of VDR ligands in the reabsorption of bile acids in the ileum of Wistar rats.

In addition to OSTα and OSTβ, multi drug resistance associated protein 2 (MRP2) and MRP3 play a role in the excretion of monovalent and conjugated bile acids across the apical and basolateral membranes of the enterocytes, respectively (5, 8, 12, 17, 31, 32, 74). Therefore, we studied the regulation of MRP2 and MRP3 transporters by VDR, FXR, GR and PXR ligands. In **chapter 5 and 6**, we showed that LCA, a VDR ligand with affinity toward FXR and PXR did not affect the MRP3 expression in ileum slices but induced MRP2 expression **(chapter 2).** These results suggest that LCA decreases the BA uptake in rat ileum by decreasing the ASBT and increases the luminal excretion of BAs in colon by induction of MRP2. Thus, in the rat LCA favours its own detoxification and transport into the lumen of the colon by inducing MRP2 but not MRP3 expression hence excretion via the faeces. In contrast, the primary BA, CDCA, which is an FXR ligand stimulates absorption

of bile salts by induction of MRP3 and OSTα-OSTβ expression and repression of MRP2 expression in rat jejunum and ileum, favouring the reclamation of bile acids in the small intestine. Also in the human intestine, LCA may induce the luminal transport of BAs by inducing MRP2 expression, whereas CDCA favours the basolateral transport of BAs by inducing MRP3 expression in ileum slices. As CDCA did not affect MRP2 expression in human ileum slices, the LCA effects are not likely to be mediated by FXR but seem to be mediated by VDR, since $1,25(OH)_2D_3$ induced MRP2 expression by 2.5-fold (un published data).

In **chapters 3 and 4**, we studied the regulation of BA transporters in rat and human liver by VDR, FXR and GR ligands. These results obtained in the human liver are summarized in the Tables 1, 2, 3 and 4. $1,25(OH)_2D_3$ significantly decreased the NTCP expression in human liver slices, without affecting the expression of BSEP, MRP2 and MRP3. The repression of NTCP by $1,25(OH)_2D_3$ can be attributed to the decrease in HNF4α, which is essential for its basal expression (26). Our results are the first to show that human NTCP is significantly decreased by the $1,25(OH)_2D_3$ and further studies are needed to elucidate whether this is indeed mediated via HNF4α down regulation. In line with earlier reports, CDCA, significantly decreased NTCP and induced BSEP expression in rat and human liver slices (2, 19), although the decrease in NTCP in human liver slices was not significant which is in line with previous data from our lab (36). Furthermore, CDCA induced MRP2 expression in rat liver slices where as in human liver slices CDCA induced MRP3 but not MRP2 which is in line with the earlier reports by Inokuchi et al. (35), who characterized a bile acid response element in the human MRP3 promoter. The GR ligands, DEX and BUD induced the NTCP expression, which is in line with the earlier reports (22). Furthermore our results show that GR ligands induce BSEP expression in rat and human liver slices, which is not reported earlier.

4 Regulation of the bile acid synthesis enzyme, CYP7A1 by VDR and FXR ligands in rat and human liver.

BAs, as mentioned in the previous section are synthesized from cholesterol (60) in the liver by a cascade of 12 reactions, initiated by the first and rate limiting step, cholesterol 7α-hydroxylation, catalyzed by CYP7A1. Recently, an altered expression of bile synthesis proteins and transporters in the liver were observed in Sprague-Dawley rats treated with $1,25(OH)_2D_3$ (14) despite the lack of localization of VDR in parenchymal cells (25). Thus, we hypothesized that changes in the expression of proteins involved in bile acid synthesis and disposition after ip administration of $1,25(OH)_2D_3$ in the rat liver (14) might be secondary to the increased bile acid flux due to increased bile acid absorption. To test this hypothesis, in **chapter 4,** we treated rat and human liver slices with the VDR ligand, $1,25(OH)_2D_3$, and also with the primary bile acid, CDCA as the FXR ligand. CDCA but not $1,25(OH)_2D_3$ significantly decreased the CYP7A1 expression with simultaneous induction of SHP in rat liver slices. Furthermore, CDCA but not $1,25(OH)_2D_3$ decreased

the expression of HNF1α, HNF4α and LRH1. Thus, in rat liver CDCA regulates CYP7A1 expression by affecting both SHP dependent pathways and SHP independent pathways, but $1,25(OH)_2D_3$ is not involved in CYP7A1 regulation. However, in human liver slices, $1,25(OH)_2D_3$ significantly decreased the expression of CYP7A1. The repression of CYP7A1 was also found after treatment with CDCA with simultaneous induction of SHP. Furthermore, $1,25(OH)_2D_3$ significantly decreased the expression of HNF4α without affecting SHP and HNF1α expression. Thus, in human liver $1,25(OH)_2D_3$ affects the SHP independent pathways. Our results showed for the first time, that $1,25(OH)_2D_3$ decreased the expression of HNF4α in human liver slices, which may contribute to a decrease in the expression of CYP7A1 (1). In conclusion, the expression of CYP7A1 was decreased by the VDR ligand, $1,25(OH)_2D_3$ in the human but not in the rat liver, whereas the FXR ligand, CDCA decreased CYP7A1 expression both in human and rat liver. This species difference in CYP7A1 regulation is consistent with the differential localization of VDR in the rat liver (exclusively in non-parenchymal cells) and human liver (non-parenchymal cells and hepatocytes). These contrasting effects of VDR and FXR ligands on the regulation of bile acid homeostasis proteins in the rat liver support the hypothesis that the decrease in CYP7A1 protein and activity by $1,25(OH)_2D_3$ in Sprague-Dawley rats, that occurred with the simultaneous induction of SHP reported by Chow et.al. (14) was secondary to the increased bile acid absorption, subsequent to the induction of ileal ASBT. However, the induction of ileal ASBT and increase in BAs in portal blood was not confirmed in our studies with Wistar rats, which can be ascribed to strain related differences and the lower dose of $1,25(OH)_2D_3$ and needs further investigation.

5 Regulation of VDR, PXR and FXR expression in rat and human ileum and liver.

In **chapter 2, 3, 4 and 7**, we also characterized the regulation of the nuclear receptors VDR, PXR and FXR by their respective ligands in rat and human intestine and liver. The GR ligands, DEX and BUD induced the expression of PXR in rat and human intestine and liver. However, the induction of PXR in rat liver seemed to be transient, observed at 8 h of incubation but not at 24 h incubation. This can be attributed to the simultaneous induction of SHP by GR ligands, as SHP is known to repress the PXR expression (55). The induction of SHP by GR ligands is not reported before. The FXR ligand, CDCA decreased the expression of PXR in rat in line with the increased SHP expression, but not in human liver slices, which is in contrast to that of earlier reports (39). Furthermore, our results in **chapter 4** suggest that CDCA decreases the expression of FXR in rat liver slices but not in human liver slices, which might probably act as feedback loop by the increased bile acid synthesis in rat liver. These results are not reported before and needs further investigation.

In chapter 7, we investigated the regulation of VDR in rat and human ileum and liver and showed that $1,25(OH)_2D_3$ and CDCA significantly induced the expression of

VDR in rat ileum and liver. Because LCA did not show these effects, these results suggest that the regulation of VDR by $1,25(OH)_2D_3$ and CDCA is not likely to be mediated by VDR or FXR activation. The involvement of Protein kinase C α (PKCα) could not be confirmed for the rat ileum but incubation of rat liver slices with the PKCα agonist, phorbol-12-myristate-13-acetate (PMA) significantly induced the VDR expression. Also in the human ileum VDR is induced by $1,25(OH)_2D_3$ and PMA but not by CDCA and LCA. Further, glucocorticoids decreased the VDR expression in rat and human liver and also in human ileum but not in rat ileum, which indicates a novel cross talk between VDR and GR.

6 Conclusions and future perspectives

In this thesis, we have demonstrated that the DMEs and DTs involved in the regulation of bile acid detoxification and synthesis, and bile acid transporters is regulated by **VDR, PXR, GR and FXR** nuclear receptors in a tissue specific manner by culturing rat and human intestine (jejunum, ileum and colon) and liver precision-cut slices in the presence of specific nuclear receptor ligands. During culturing of the slices in the medium, the expression of DMEs and DTs are altered, showing both up and down regulation **(chapter 2, 3, 4, 5, 6 and 7)**. As the mRNA expression of DMEs and DTs are regulated *in vivo* by natural inducers or repressors these changes can be explained by lack of these natural regulatory factors. For example the lack of bile acids in our culture medium can explain the changes in MRP2 and MRP3 expression in rat intestinal slices during control incubations **(chapter 3)**. However the slices remain responsive to inducers. Therefore, for the characterization and understanding of the role of NRs in the regulation DMEs and DTs in principle it might be useful to modify the culture medium to minimize the alteration of DMEs and DTs in the slices. During incubation the intestinal slices are equally exposed to the inducers from both the apical (luminal) and basolateral (blood) side, which is different from the *in vivo* situation, where orally taken drugs and endogenous bile acids are present at high concentrations in the intestinal lumen and the consequences need to be evaluated. Furthermore, significant interspecies differences were observed in the regulation of DMEs, DTs and NRs between rat and human intestine and liver and thus extrapolation of data from preclinical species to man is hazardous and underline the necessity of relevant *in vitro* models for human studies. The results presented in this thesis are based on the observed changes in mRNA levels of DMEs, DTs and NRs involved in bile acid synthesis, transport and detoxification in response to various nuclear receptor ligands measured by quantitative real time PCR (qRT-PCR). Although qRT-PCR is a powerful technique in measuring the changes in the gene expression, the changes in the mRNA expression levels might not always lead to the proportional changes in the protein expression and hence the activity. In future experiments, the changes in gene expression induced by the various nuclear receptor ligands need to be characterized at the level of protein either by western blot or by immunohistochemistry. To further gain insight in the role of various nuclear factors regulating the expression of genes at the transcription level, siRNA transfections in the

slices can be performed to momentarily knock out the particular transcription factor and to study the effects on a particular pathway.

Table 1 Summary of the effects of 1,25(OH)$_2$D$_3$ (200 nM) on the expression genes in human livers; n= 4-5 human liver donors; criteria for induction and repression are 1.5-fold and 0.7-fold, respectively.

Human livers			HL1	HL2	HL5	HL6	HL3	HL4	HL7	Mean	S.E.M.	S *t*-Test
Gender			Female	N/A	Male	N/A	Female	Female	Female			
Age			54	N/A	65	N/A	72	64	42			
ATP pmol / µg of protein ± SD			*10.4 ± 1.5	*5.7 ± 1.9	*12.1 ± 1.0	11.1 ± 0.9	*3.3 ± 1.2	*9.7 ± 1.8	ND			
VDR (ΔC_T)			+ ve	+ ve	+ ve	+ ve	- ve	- ve	ND			
Gene												
1,25(OH)$_2$D$_3$	↑	CYP3A4	2.3	↔	1.5	1.9	0.3	ND	↔	1.30	0.28	0.294
	↓	CYP7A1	0.7	↔	0.7	↔	0.3	ND	↔	0.67	0.08	0.002
	↔	SHP	↔	1.5	0.27	↔	↔	ND	↔	0.91	0.15	0.556
	↔	HNF1α	↔	↔	↔	↔	1.5	ND	0.6	0.99	0.13	0.925
	↓	HNF4α	↔	↔	0.5	↔	0.5	ND	0.7	0.72	0.08	0.004
	↔	LXRα	0.7	↔	0.7	ND	2.16	ND	ND	1.12	0.31	0.640
	↔	PXR	↔	↔	↔	ND	0.66	ND	ND	0.82	0.06	0.004
	↔	FXR	0.7	↔	0.7	ND	↔	ND	ND	0.84	0.09	0.066
	↔	VDR	↔	0.5	↔	↔	NDE	NDE	ND	0.90	0.17	0.596
	↔	BSEP	0.5	0.7	↔	↔	1.96	ND	1.5	1.13	0.21	0.551
	↔	NTCP	0.4	↔	↔	↔	0.4	ND	↔	0.78	0.13	0.100
	↔	MRP2	0.5	↔	↔	↔	1.5	ND	↔	1.01	0.14	0.950
	↔	MRP3	↔	↔	0.7	0.7	NDE	ND	1.38	0.98	0.13	0.840
	↔	ASBT	↔	0.6	↔	ND	0.6	ND	ND	0.92	0.19	0.580
	↔	OSTα	0.5	2.9	0.5	ND	0.4	ND	ND	1.07	0.60	0.875
	↓	OSTβ	0.6	↔	0.4	ND	0.4	ND	ND	0.69	0.21	0.074

HL-human liver; ND-not done; NDE-not detectable; N/A – not available; TX – transplantation liver; PH partial hepatectomy liver; ↔ - No effect; ↓-repression; ↑-induction; "" data is taken from Khan et al.(2009a); All values are expressed as fold induction with respect to their solvent incubated controls.*

Table 2 Summary of the effects of chenodeoxycholic acid (CDCA) on the expression genes in human livers; n= 4-5 human liver donors; criteria for induction and repression are 1.5-fold and 0.7-fold, respectively.

Human livers			HL1	HL2	HL5	HL3	HL4	Mean	S.E.M.	S *t*-Test
Gender			Female	N/A	Male	Female	Female			
Age			54	N/A	65	72	64			
ATP pmol / µg of protein ± SD			*10.4 ± 1.5	*5.7 ± 1.9	*12.1 ± 1.0	*3.3 ± 1.2	*9.7 ± 1.8			
VDR (ΔC_T)			11.7	16.19	14.8	NDE	NDE			
Gene										
CDCA	↑	CYP3A4	1.7	1.5	1.8	2.7	9.0	3.34	1.43	0.140
	□	CYP7A1	0.7	0.07	0.3	↔	0.1	0.45	0.21	0.033
	↑	SHP	3.4	2.1	↔	4.76	6.57	3.55	0.99	0.033
	↔	HNF1α	↔	0.7	0.1	↔	ND	0.77	0.23	0.281
	↔	HNF4α	0.6	↔	0.1	2.4	ND	0.98	0.50	0.717
	↓	LXRα	↔	↔	0.6	0.5	↔	0.97	0.17	0.846
	↔	PXR	0.7	0.7	2.8	2.2	0.3	1.35	0.48	0.485
	↔	FXR	0.7	↔	↔	↔	↔	0.92	0.11	0.500
	↔	VDR	↔	↔	0.3	NDE	NDE	0.748	0.22	0.293
	↑	BSEP	2.3	↔	6.6	1.9	2.1	2.77	0.98	0.110
	↓	NTCP	0.4	↔	0.5	1.9	0.3	0.78	0.29	0.473
	↔	MRP2	↔	0.7	↔	2.14	↔	1.09	0.30	0.456
	↑	MRP3	↔	2.3	1.5	NDE	2.1	1.80	0.24	0.007
	↔	ASBT	↔	1.5	0.2	↔	↔	1.03	0.22	0.909
	↑	OSTα	10.7	24.3	11.9	4.8	25.2	15.38	4.01	0.007
	↑	OSTβ	117	91	132	14	204	112	30.69	0.007

HL-human liver; ND-not done; NDE-not detectable; N/A – not available; TX – transplantation liver; PH partial hepatectomy liver; ↔ - No effect; ↓-repression; ↑-induction; "" data is taken from Khan et al.(2009a); All values are expressed as fold induction with respect to their solvent incubated controls*

Table 3 Summary of the effects of dexamethasone (DEX) on the expression genes in human livers; n= 4-5 human liver donors; criteria for induction and repression are 1.5-fold and 0.7-fold, respectively.

Human livers			HL1	HL2	HL5	HL3	HL4	Mean	S.E.M.	S *t*-Test
Gender			Female	N/A	Male	Female	Female			
Age			54	N/A	65	72	64			
ATP pmol / μg of protein ± SD			*10.4 ± 1.5	*5.7 ± 1.9	*12.1 ± 1.0	*3.3 ± 1.2	*9.7 ± 1.8			
VDR (ΔC_T)			11.7	16.19	14.8	NDE	NDE			
Gene										
DEX	↑	CYP3A4	17.6	4.0	15.0	1.7	9.1	9.52	3.05	0.023
	↔	CYP7A1	1.9	0.3	0.2	1.7	0.1	0.83	0.39	0.677
	↔	SHP	↔	↔	2.8	↔	6.8	2.52	1.13	0.215
	↑	HNF1α	↔	↔	1.6	1.7	ND	1.35	0.20	0.079
	↑	HNF4α	1.9	↔	6.5	3.9	ND	3.31	1.24	0.071
	↔	LXRα	↔	↔	↔	↔	1.5	1.02	0.15	0.908
	↑	PXR	↔	↔	2.3	↔	1.6	1.5	0.21	0.043
	↔	FXR	0.3	↔	0.4	2.3	↔	1.05	0.37	0.899
	↓	VDR	0.4	0.4	0.2	ND	NDE	0.31	0.05	0.000
	↑	BSEP	8.7	2.3	15.2	3.3	3.6	6.61	2.40	0.048
	↑	NTCP	6.5	1.5	4.2	2.3	↔	3.04	1.03	0.083
	↔	MRP2	↔	1.9	0.6	↔	↔	1.2	0.22	0.387
	↔	MRP3	0.5	↔	2.0	ND	0.5	0.95	0.35	0.861
	↑	ASBT	3.6	2.0	1.5	2.2	1.9	2.23	0.36	0.010
	↓	OSTα	0.7	0.2	0.5	0.6	0.6	0.51	0.08	0.000
	↑	OSTβ	1.8	5.4	↔	↔	4.6	2.71	0.95	0.109

HL-human liver; ND-not done; NDE-not detectable; N/A – not available; TX – transplantation liver; PH partial hepatectomy liver; ↔ - No effect; ↓-repression; ↑-induction; "" data is taken from Khan et al.(2009a); All values are expressed as fold induction with respect to their solvent incubated controls.*

Table 4 Summary of the effects of lithocholic acid (LCA) on the expression genes in human livers; n= 4-7 human liver donors; criteria for induction and repression are 1.5-fold and 0.7-fold, respectively.

Human livers			HL1	HL2	HL5	HL6	HL3	HL4	HL7	Mean	S.E.M.	*p*
Gender			Female	N/A	Male	N/A	Female	Female	Female			
Age			54	N/A	65	N/A	72	64	42			
ATP pmol / μg of protein ± SD			*10.4 ± 1.5	*5.7 ± 1.9	*12.1 ± 1.0	11.1 ± 0.9	*3.3 ± 1.2	*9.7 ± 1.8	ND			
VDR (ΔC_T)			11.7	16.2	14.8	16.2	NDE	NDE	ND			
Gene												
LCA	↔	CYP3A4	0.6	0.6	1.8	0.5	2.2	3.2	2.0	1.54	0.38	0.175
	↔	CYP7A1	↔	↔	0.3	0.5	↔	0.1	1.9	0.90	0.25	0.683
	↔	SHP	↔	0.7	0.7	↔	2.6	3.1	↔	1.40	0.38	0.312
	↔	HNF1α	↔	↔	0.3	↔	↔	ND	↔	0.78	0.10	†0.042
	↔	HNF4α	↔	0.4	0.2	0.4	2.4	ND	0.4	0.80	0.34	0.523
	↔	LXRα	0.6	0.7	0.4	ND	↔	↔	ND	0.77	0.19	0.166
	↔	PXR	0.7	0.3	1.7	ND	1.5	0.4	ND	0.93	0.30	0.794
	↔	FXR	0.6	↔	0.5	ND	↔	↔	ND	0.86	0.16	0.336
	□	VDR	↔	0.5	0.5	0.3	NDE	NDE	ND	0.51	0.12	0.017
	↔	BSEP	0.4	↔	1.7	↔	0.7	0.2	↔	0.84	0.19	0.413
	↔	NTCP	0.3	0.30	↔	0.1	2.3	0.1	2.2	0.86	0.36	0.712
	↔	MRP2	0.5	↔	0.5	↔	↔	↔	1.5	1.03	0.16	0.867
	↔	MRP3	↔	↔	↔	↔	NDE	↔	↔	1.03	0.09	0.741
	↔	ASBT	↔	0.6	0.16	ND	↔	2.3	ND	1.08	0.40	0.796
	↑	OSTα	1.5	2.6	↔	ND	3.9	5.2	ND	2.87	0.85	0.014
	↑	OSTβ	2.6	1.7	2.5	ND	6.2	4.2	ND	3.42	0.89	0.004

HL-human liver; ND-not done; NDE-not detectable; N/A – not available; TX – transplantation liver; PH partial hepatectomy liver; ↔ - No effect; ↓-repression; ↑-induction; "" data is taken from Khan et al.(2009a); All values are expressed as fold induction with respect to their solvent incubated controls.† indicates all samples showed down regulation but to a different extent, which is outside the criteria for induction and repression.*

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Chapter 9
Summary

The research described in this thesis is focused on the regulation of drug metabolizing enzymes (DMEs) and drug transporters (DTs) that are involved in drug metabolism and transport as well as in bile acid synthesis, detoxification and transport in the human and rat intestine and liver. To predict drug metabolism and transport in man, animal studies are widely applied and among the rodents, the rat is the most preferred preclinical animal species used in the pharmacological and toxicological evaluation of new chemical entities (NCE) in drug discovery. However interspecies differences make this extrapolation hazardous and unreliable. Moreover the expression of DMEs and DTs is subject to regulation by ligand-activated nuclear receptors (NRs), which also exhibit profound interspecies differences. The most important NRs regulating the expression of DME's and DT's are the pregnane X receptor (PXR), the constitutive androstane receptor (CAR), the glucocorticoid receptor (GR), the vitamin D receptor (VDR), the aryl hydrocarbon receptor (Ahr) and nuclear factor E2-related factor 2 (Nrf2).

Data on the regulation of the expression of DMEs and DTs in animals can be obtained from *in vivo* studies but in man *in vivo* data is difficult to obtain. For human studies, occasionally tissue from patients is studied but it remains undetermined to what extent confounding factors of the disease interfere with the results, and moreover secondary effects of the ligands cannot be discriminated from primary effects. Therefore *in vitro* methods are required to study the direct effects of drugs on the regulation of DMEs and DTs. The available *in vitro* methods to study the regulation of DMEs and DTs in human liver and intestine are primary cells (hepatocytes and enterocytes), and immortalized cell lines (HepG2 and Caco-2). However, the primary cells are unstable with respect to the expression of DME's, DT's and NRs whereas immortalized cell lines exhibit inadequate expression of DME's, DT's and NRs. In this thesis, precision-cut liver slices (PCLS) and the recently validated precision-cut intestinal slices (PCIS) are used as an *in vitro* model to study the regulation of DME's and DT's in rat and human intestine and liver as well as the species-specificity and inter-organ differences herein. As these functions vary along the length of the intestine, slices were prepared from jejunum, ileum and colon.

The main focus of the thesis was on the VDR mediated effects by calcitriol ($1,25(OH)_2D_3$) and lithocholic acid (LCA) on the regulation of DME's and DT's. Further, to obtain more insight in the mechanism, the effects of the VDR ligands were compared with those of specific ligands for other nuclear receptors, chenodeoxycholic acid (CDCA) and GW4064 for FXR, and pregnenolone-16α carbonitrile (PCN) for PXR, budesonide (BUD) for GR and dexamethasone (DEX) for GR and PXR.

In **chapter 1**, the interspecies and inter-organ differences in the expression and regulation of DME's and DT's and the currently available models to study their impact on drug metabolism are summarized.

In **chapter 2**, the mRNA expression of the nuclear receptors VDR, PXR and FXR in the different regions of the rat and human intestine was characterized. In addition, the role of the VDR-, PXR-, GR- and FXR-specific ligands: $1,25(OH)_2D_3$, LCA, PCN, DEX, BUD and CDCA in the regulation of the mRNA expression of cytochrome P450 (CYP P450) 3A isozymes was studied in different regions of the rat intestine and liver, and in the human ileum and liver using precision-cut tissue slices. The CYP3A isozymes are responsible for the metabolism of 60% of the marketed drugs and of a number of endogenous compounds such as bile acids. In the rat intestine, CYP3A1 expression was found to be highly inducible by the VDR ligand, and to a lesser extent by PXR and GR ligands. The induction showed a gradient along the length of the intestine which was different for the different ligands but did not correlate with the gradient of the respective NRs. Furthermore, CYP3A2 expression was found to be exclusively regulated by the VDR ligand in the rat ileum only. CYP3A9 expression in the liver and in all regions of the intestine appeared to be mainly regulated by PXR and GR ligands but not by the VDR ligand. In contrast, the VDR ligand did not affect the expression of CYP3A isozymes in the rat liver. This organ specificity can be explained by the specific localization of VDR in the bile duct epithelial cells. In the rat liver VDR is not expressed in the hepatocytes, where CYP3A isozymes are highly expressed. In contrast to the rat CYP3A isozymes, human CYP3A4 was induced by the VDR ligand in ileum and liver, which can be explained by the observation that in the human liver VDR is expressed in the hepatocytes along with CYP3A4, which was confirmed at the level of protein by immunohistochemistry **(chapter 4).** These VDR ligand mediated *in vitro* effects on the regulation of CYP3A isozymes in the rat intestine and liver were confirmed *in vivo* by treating the rats with $1,25(OH)_2D_3$ **(chapter 5)**.

In **chapter 3**, the effects of another endogenous VDR ligand, LCA, on the regulation of CYP3A isozymes in the rat intestine and liver, and the human ileum and liver at the level of mRNA were investigated. LCA is a toxic bile acid formed by bacterial biotransformation of the primary bile acid CDCA in the terminal part of the ileum. In contrast to $1,25(OH)_2D_3$, LCA is also a ligand for PXR and FXR. Similar to $1,25(OH)_2D_3$ LCA induced the expression of CYP3A1 along the length of the intestine with the highest induction in ileum slices, and CYP3A2 in ileum slices only. Furthermore, in contrast to $1,25(OH)_2D_3$, LCA also induced the expression of CYP3A9 in liver slices and along the length of the intestine with a moderately higher induction in the colon slices, which is presumably mediated by PXR. LCA induced CYP3A4 in human ileum slices, in contrast to $1,25(OH)_2D_3$, LCA did not affect the CYP3A4 expression in human liver slices. Furthermore, as LCA is also an FXR ligand, and FXR is known to play an important role in the regulation of bile acid homeostasis, the direct effects of LCA on the regulation of a bile acid synthesis enzyme, bile acid transporters and nuclear receptors were studied in rat and human liver slices and compared with the effects of CDCA, a high affinity FXR ligand **(chapter 4)** and these results are summarized in **chapter 8.**

In **chapter 4,** the influence of the VDR ligand, $1,25(OH)_2D_3$ on the regulation of bile acid homeostasis was compared with that of the FXR ligand, CDCA by measuring the mRNA expression of the bile acid transporters, the rate limiting enzyme in bile acid synthesis, CYP7A1 as well as the NRs involved in this regulation in rat and human liver. The VDR ligand, $1,25(OH)_2D_3$ decreased the expression of CYP7A1 and the basolateral transporter, sodium dependent taurocholate co-transporting protein (NTCP) expression in human liver slices but not in rat liver slices, without affecting the expression of the canalicular bile acid transporters BSEP and MRP2 in human and rat liver slices. In line with these findings, $1,25(OH)_2D_3$ decreased the expression of HNF4α, which is reported to be essential for the basal expression of NTCP in human but not in rat liver. As expected CDCA decreased the NTCP and induced the BSEP expression in rat and human liver slices, although the decrease in NTCP expression in human liver slices was not significant, which is in line with previous data from our lab. These data confirms the intactness of the FXR pathway in the liver slices during incubation. The species specific differences in the VDR effects in the regulation of CYP7A1 and NTCP in human and rat liver slices is attributed to the species differences in the cellular localization of VDR, which is expressed in human hepatocytes but not in the rat hepatocytes, which is confirmed at the level of protein by immunohistochemistry.

In chapter 5, the VDR, FXR and GR mediated regulation of the apical sodium dependent bile acid export transporter (ASBT) and the NRs regulating its expression in rat and human ileum and liver precision-cut tissue slices was characterized at the level of mRNA. The VDR ligand, $1,25(OH)_2D_3$ significantly decreased the expression of ASBT mRNA in rat ileum and liver slices, and in the human ileum but not in the human liver. The $1,25(OH)_2D_3$ mediated ASBT repression in rat ileum slices is in contrast to the data of Chen et al**. (*Mol Pharmacol 69: 1913-1923, 2006)***, who showed a positive VDRE in rat ASBT promoter and induction of ASBT mRNA expression and activity in the ileum of Sprague-Dawley rats with high doses of $1,25(OH)_2D_3$. Interestingly, we found a repression of HNF1α in rat ileum slices treated with $1,25(OH)_2D_3$, which may explain the down regulation of ASBT since HNF1α is essential for the basal expression of ASBT. These results were further confirmed with the natural VDR ligand LCA, which also showed a significant repression of ASBT in rat ileum with concomitant repression of HNF1α. Like in rat ileum slices, $1,25(OH)_2D_3$ decreased the ASBT expression in rat liver slices, in line with the co-localization of VDR and ASBT in the biliary epithelial cells. $1,25(OH)_2D_3$ also decreased the ASBT expression in the human ileum slices, whereas in the human liver slices ASBT expression remained unaltered, which is as yet unexplained but indicates species and organ specific differences in the regulation of ASBT in rat and human ileum and liver. However, the *in vitro* results of repression of ASBT were not found in Wistar rats *in vivo* treated with $1,25(OH)_2D_3$, where ASBT remained unaltered. These differences might be attributed to a lower exposure of the ileocytes to the effective concentration of $1,25(OH)_2D_3$ in ileum *in vivo* after the intraperitoneal administration, as we also found a much lower and highly variable CYP3A1 induction *in vivo* as compared to the high and

consistent induction *in vitro* in ileum slices cultured in the presence of $1,25(OH)_2D_3$ **(Chapter 2**). Also LCA decreased the expression of ASBT in the ileum of man and rat, which may be a VDR and/or an FXR effect as the FXR ligand CDCA also reduced the ASBT expression in the human ileum but not in rat ileum. In accordance with in vivo data, the GR ligands, DEX and BUD induced the ASBT expression in rat and human ileum slices with simultaneous induction of HNF1α, showing the viability of the slice model to show an induction of ASBT.

In **chapter 6,** the regulation of the basolateral bile acid transporter, organic solute transporter (OST) consisting of two half transporters, α and β (OSTα and OSTβ) involved in the absorption of bile acids in the rat and human ileum and by the cholangiocytes in the liver by VDR, FXR, GR and PXR nuclear receptor ligands was studied using precision cut rat and human intestinal and liver slices. The rat but not the human OSTα and OSTβ genes in the ileum were negatively regulated by the VDR ligands, $1,25(OH)_2D_3$ and LCA. This repression of rat OSTα and OSTβ genes by $1,25(OH)_2D_3$ led to the postulation of a negative VDRE in the rat promoters, which remains to be confirmed. OSTα and OSTβ are positively regulated by the GR ligand, DEX but not by the PXR ligand. Further studies are needed to evaluate the role of VDR ligands in the reabsorption of bile acids in the ileum of Wistar rats.

In chapter 7, we investigated the regulation of VDR in rat and human ileum and liver and showed that $1,25(OH)_2D_3$ and CDCA significantly induced the expression of VDR in rat ileum and liver. Because LCA, a known VDR ligand did not show these effects, these results suggest that the regulation of VDR by $1,25(OH)_2D_3$ and CDCA is not likely to be mediated by VDR or FXR activation. The involvement of proteinkinase Cα (PKCα) could not be confirmed for the rat ileum but incubation of rat liver slices with the PKCα agonist, phorbol-12-myristate-13-acetate (PMA) significantly induced the VDR expression. Also in the human ileum VDR is induced by $1,25(OH)_2D_3$ and PMA but not by CDCA and LCA. Further, glucocorticoids decreased the VDR expression in the rat and human liver and also in the human ileum but not in the rat ileum, which indicates a novel cross talk between VDR and GR.

In **Chapter 8**, the results of **chapters 2, 3, 4, 5, 6 and 7** were discussed and the major conclusions were summarized.

In summary, we have clearly demonstrated in this thesis that the enzymes and transporters involved in drug metabolism, bile acid detoxification and synthesis, and bile acid transport are regulated by the nuclear receptors **VDR, PXR, GR and FXR** in a tissue specific manner by culturing rat and human intestine (jejunum, ileum and colon) and liver precision-cut slices in the presence of specific nuclear receptor ligands. This *in vitro* model was found to reflect the *in vivo* established regulations properly. In contrast to *in vivo* where confounding secondary reactions may influence the regulation, the slice model

allows the study of direct effects of a ligand in different organs under identical circumstances, taking into account not only binding to the NR but also uptake, metabolism and excretion of the ligand as well as possible influences of co-activators and repressors. Furthermore, significant interspecies differences were observed in the regulation of DMEs, DTs and NRs between rat and human intestine and liver. This implicates that extrapolation of data from preclinical species to man is hazardous and underlines the necessity of relevant *in vitro* models for human studies. *In vitro* research with human tissue slices contributes to obtain human specific data and to reduce the number of experimental animals.

Chapter 10
Samenvatting (Dutch Summary)

Het onderzoek beschreven in dit proefschrift was erop gericht om de regulatie van de expressie van eiwitten die betrokken zijn bij het metaboliseren en het transport van geneesmiddelen en galzouten in de lever en de darm van de mens en de rat te bestuderen. Voor het voorspellen van metabolisme en transport van geneesmiddelen worden vooral gegevens gebruikt die verkregen worden uit dierproeven. Knaagdieren en met name ratten worden veel gebruikt voor het preklinisch onderzoek naar de farmacologische en toxicologische eigenschappen, inclusief het metabolisme en transport, van nieuw te ontwikkelen geneesmiddelen. Maar door de grote verschillen tussen mens en dier zijn deze voorspellingen vaak onnauwkeurig en onbetrouwbaar. Niet alleen de expressie van de metabole enzymen en de transporters zelf vertonen grote interspecies verschillen, ook bij de expressie van de nucleaire receptoren die betrokken zijn bij die regulatie worden grote verschillen tussen mens en dier gevonden. De belangrijkste nucleaire receptoren (NR) die betrokken zijn bij de expressie van geneesmiddel metaboliserende enzymen (GME) en transporters (GT) zijn de pregnan-X-receptor (PXR), de constitutieve androstaan receptor (CAR) de glucocorticoid receptor (GR), de vitamine D receptor (VDR), de aryl koolwaterstof receptor (Ahr) en de nuclear factor 2- gerelateerde factor 2 (Nrf2).

Gegevens over de regulatie van de GMEs en GTs in dieren kunnen worden verkregen uit in vivo dierproeven, maar bij de mens is dit veel moeilijker te onderzoeken. Soms kan hiervoor weefsel van patiënten worden gebruikt dat bij een operatie wordt verwijderd, maar het blijft moeilijk vast te stellen in hoeverre de ziekte invloed heeft op de gemeten parameters. Bovendien is het vrijwel onmogelijk om indirecte effecten van geneesmiddelen van directe effecten te onderscheiden. Om die directe effecten van geneesmiddelen op de regulatie van GMEs en GTs te meten zijn in vitro experimenten vereist. Hiervoor worden vaak geïsoleerde menselijke levercellen (hepatocyten) en darmcellen (enterocyten) gebruikt naast geïmmortaliseerde cellijnen zoals HepG2 cellen en Caco-2 cellen. Echter in de verse cellen is de expressie van de GMEs, GTs an NRs niet stabiel, terwijl die expressie in de cellijnen vaak niet overeenkomt met die in het weefsel. In het onderzoek beschreven in dit proefschrift worden precies gesneden leverslices en darmslices gebruikt als *in vitro* model. Daarmee is de regulatie van GMEs en GTs bestudeerd in ratten en humane lever en darm om zo de species verschillen en verschillen tussen de organen te onderzoeken. Omdat deze functies verschillen in de verschillende segmenten van de darm zijn slices gemaakt van jejunum, ileum en colon.

De nadruk van het onderzoek lag op de effecten van de liganden van de Vitamine D receptor $1,25(OH)_2D_3$, de actieve vorm van Vitamine D, en het galzout lithocholzuur (LCA). Om meer inzicht te verkrijgen in de specificiteit van deze regulatie werden de effecten van deze VDR liganden vergeleken met die van specifieke liganden van de andere NRs, zoals de FXR liganden chenodeoxycholzuur (CDCA) en GW4064 , de PXR liganden pregnenolone-16α carbonitril (PCN), het GR ligand budesonide (BUD) en dexamethason als ligand voor zowel GR als PXR.

In **hoofdstuk 1** wordt een overzicht gegeven van wat er bekend is over de orgaan en species verschillen in de expressie van de GMEs en GTs en van de beschikbare in vitro modellen die gebruikt worden om die regulatie te bestuderen.

In **hoofdstuk 2** werd eerst de mRNA expressie van de NRs VDR, PXR en FXR gekarakteriseerd in de verschillende delen van de darm (jejunum, ileum en colon) van de rat en de mens. Vervolgens werd de invloed van de liganden voor de verschillende NRs op de expressie van de cytochroom P450 3A (CYP3A) isoenzymen gemeten in slices van de lever en de verschillende darmsegmenten van de mens en de rat. Deze CYP3A isoenzymen zijn verantwoordelijk voor het metaboliseren van ca 60% van alle geneesmiddelen alsmede van een aantal endogene stoffen zoals galzouten. In de rattendarm werd een sterke inductie gevonden van CYP3A1 door het VDR ligand $1,25(OH)_2D_3$ en een geringere inductie door de PXR en GR liganden. Deze inductie was verschillend in de verschillende darmsegmenten waarbij de richting van de gradient verschilde per ligand. Deze verschillen in mate van regulatie waren niet gecorreleerd met de expressie van de betrokken NRs. CYP3A2 werd alleen geïnduceerd door het VDR ligand en deze regulatie werd alleen in het ileum van de rat waargenomen. De expressie van CYP3A9 bleek voornamelijk te worden gereguleerd door PXR en GR liganden maar niet door het VDR ligand zowel in alle segmenten van de darm als in de lever. In de lever werd geen effect van $1,25(OH)_2D_3$ gemeten op de expressie van de CYP3A isoenzymen. Dit verschil tussen lever en darm kan verklaard worden door het feit dat in de rattenlever VDR nauwelijks voorkomt in de hepatocyten, waar de meeste CYP3A is gelokaliseerd, maar voornamelijk is gelokaliseerd in de galgangepitheelcellen. Daarentegen wordt bij de mens CYP3A4 door $1,25(OH)_2D_3$ geïnduceerd zowel in de darmslices als in de leverslices, aangezien in de humane hepatocyten wel VDR aanwezig is, hetgeen werd bevestigt op eiwitniveau m.b.v. immunohistochemie. (**Hoofdstuk4**) Deze *in vitro* gevonden effecten werden bevestigd in experimenten waarbij ratten *in vivo* $1,25(OH)_2D_3$ kregen toegediend (**hoofdstuk 5**).

In **hoofdstuk 3** worden de effecten beschreven van een ander endogeen VDR ligand, LCA op de regulatie van CYP3A isoenzymen in de lever en de darm van de rat en de mens. LCA is een toxisch galzout dat door bacteriën in het ileum wordt gevormd uit het primaire galzout CDCA. In tegenstelling tot $1,25(OH)_2D_3$ is LCA ook een ligand voor FXR en PXR. Net als $1,25(OH)_2D_3$ induceerde LCA de expressie van CYP3A1 in alle segmenten van de darm waarbij het grootste effect in het ileum werd gevonden. Ook werd uitsluitend in het ileum CYP3A2 geïnduceerd. In tegenstelling tot $1,25(OH)_2D_3$ induceerde LCA ook de expressie van CYP3A9 in slices van de lever en van alle darmsegmenten, hetgeen waarschijnlijk aan een PXR effect kan worden toegeschreven. LCA induceerde de expressie van CYP3A4 in de humane ileum slices maar in tegenstelling tot $1,25(OH)_2D_3$ niet in de humane leverslices. Omdat LCA ook een FXR ligand is en FXR een belangrijke rol speelt in de galzout homeostasis, werden ook de effecten van LCA op de regulatie van CYP7A1, betrokken bij de synthese van galzouten, en van galzout transporters bestudeerd

en vergeleken met de effecten van CDCA, een FXR ligand met hoge affiniteit (**Hoofdstuk4**). Deze resultaten staan samengevat in **hoofdstuk 8**.

In **hoofdstuk 4** werd de invloed van de VDR ligand $1,25(OH)_2D_3$ op de regulatie van de galzout homeostase in de lever vergeleken met die van de FXR ligand CDCA. Daarvoor werden de expressie van CYP7A1, betrokken bij de synthese van galzouten, en van galzouttransporters en de NR betrokken bij deze regulatie bestudeerd. $1,25(OH)_2D_3$ verlaagde de expressie van CYP7A1 en van de basolaterale transporter NTCP (de natrium afhankelijke taurocholaat cotransporter) in humane maar niet in rattenleverslices. Deze resultaten zijn in overeenstemming met de gevonden verlaagde HNF4α expressie, waarvan bekend is dat het essentieel is voor de basale expressie van NTCP in de humane maar niet in de rattenlever. De expressie van de galzoutexporttransporters BSEP en MRP2 werd niet beïnvloed. Zoals verwacht, verlaagde CDCA de NTCP expressie en verhoogde het de BSEP expressie, hoewel de verlaagde NTCP expressie in de humane lever niet significant was, zoals ook eerder was gevonden in ons lab. Deze uitkomsten bevestigen dat de effecten van FXR activatie in de slices goed overeenkomen met de effecten die in vivo worden gevonden. De speciesverschillen in de effecten van VDR activatie kunnen worden toegeschreven aan de afwezigheid van de VDR expressie in rattenhepatocyten, die werd bevestigd op eiwitniveau met immunohistochemische kleuring.

In **hoofdstuk 5** werd de VDR, FXR en GR gemedieerde regulatie van de apicale natrium afhankelijke galzouttransporter ASBT en de daarbij betrokken NR gekarakteriseerd in de darm en de lever van mens en rat. De VDR ligand $1,25(OH)_2D_3$ had een verlagend effect op de expressie van ASBT mRNA in de rattendarm en–lever en in de humane darm maar niet in de humane lever. Deze resultaten zijn in tegenspraak met die van Chen et al **(Mol. Pharmacol 69: 1913-1923, 2006)** die een positieve VDR responsive element in de ASBT promotor aantoonde en inductie van ASBT in de ileum van Sprague Dawley ratten na intraperitoneale injectie van hoge doses van $1,25(OH)_2D_3$. De verlaging van ASBT in onze experimenten ging gepaard met een verlaging van HNF1α, een NR die essentieel is voor de basale expressie van ASBT. Ook LCA veroorzaakte een verlaging van ASBT en van HNF1α. Ook in de rattenlever werd ASBT verlaagd door $1,25(OH)_2D_3$, hetgeen te verklaren is doordat VDR en ASBT beide in de cholangiocyten tot expressie komen. $1,25(OH)_2D_3$ verlaagde ook de ASBT expressie in de humane ileum maar niet in de humane leverslices. Voor dit species-en orgaanverschil hebben we nog geen verklaring kunnen vinden. De in vitro gevonden verlaging van de expressie van ASBT in de ratten darm werd niet gevonden bij *in vivo* experimenten waarbij we Wistar ratten $1,25(OH)_2D_3$ intraperitoneaal toedienden, en waarbij de ASBT expressie niet veranderde. Dit zou kunnen worden veroorzaakt doordat de enterocyten in het ileum bij deze experimenten waarschijnlijk aan een lagere concentratie van $1,25(OH)_2D_3$ werden blootgesteld dan de slices, omdat ook de verhoging van de CYP3A1 expressie *in vivo* veel lager was dan *in vitro* **(zie hoofdstuk 2)**. LCA verlaagde eveneens de ASBT expressie in de humane en rattendarm, hetgeen een VDR maar ook een FXR effect zou kunnen zijn aangezien ook

FXR activatie door CDCA een verlaging van ASBT in de humane ileum maar niet in de rattenileum veroorzaakte. Net als *in vivo* was aangetoond, induceerden de GR liganden Dex en BUD de ASBT expressie samen met de HNF1α expressie, hetgeen aantoont dat ASBT inductie in slices kan plaatsvinden.

In **hoofdstuk 6** werd de regulatie van de basolaterale galzout export transporter Organic Solute Transporter (Ost) door VDR, FXR, GR en FXR liganden bestudeerd. Ost bestaat uit twee halftransporters, Ostα en Ostβ en is betrokken bij de uitscheiding van galzouten naar het bloed vanuit de enterocyten en de cholangiocyten. In het ileum van de rat maar niet van de mens werd een repressie van de expressie van Ostα en Ostβ gevonden door de VDR liganden $1,25(OH)_2D_3$ en LCA. Dit zou verklaard kunnen worden door een negatieve VDRE te postuleren. Zoals verwacht werden Ostα en Ostβ positief gereguleerd door de GR ligand DEX, maar er werd geen regulatie gevonden door de PXR liganden PCN. Om de rol van VDR bij de regulatie van ASBT nader te onderzoeken is meer onderzoek nodig.

In **hoofdstuk 7** onderzochten we de regulatie van de expressie van de nucleaire receptor VDR. Zowel $1,25(OH)_2D_3$ als CDCA induceerden de expressie van VDR in de rattendarm en –lever. Maar omdat LCA VDR niet induceerde suggereren deze resultaten dat deze regulatie door CDCA en $1,25(OH)_2D_3$ niet door FXR of VDR wordt gemedieerd. De betrokkenheid van proteïnekinase Cα (PKCα) kon niet worden anagetoond in de darmslices maar wel in de leverslices waar de PKCα agonist phorbol-12-myristate-13 acetate (PMA) de VDR expressie significant verhoogde. Ook in de humane darm werd VDR opgereguleerd door PMA en $1,25(OH)_2D_3$, maar niet door CDCA en LCA. Ook DEX verlaagde de VDR expressiein de humane darm en lever en in de rattenlever. Deze cross-talk tussen GR en VDR was nog niet eerder aangetoond.

In **hoofdstuk 8** worden de belangrijkste resultaten uit de hoofdstukken 2-7 samengevat en bediscussieerd.

In dit proefschrift is aangetoond dat de regulatie van de enzymen en transporters betrokken bij geneesmiddel- en galzoutmetabolisme en transport door de nucleaire receptoren VDR, FXR, PXR en GR weefselspecifiek is. Het gebruikte *in vitro* model bleek regulatie, voor zover die bekend was uit *in vivo* studies, adequaat te reflecteren. In tegenstelling tot de situatie in een *in vivo* experiment waar secundaire effecten van liganden kunnen optreden die moeilijk van directe effecten kunnen worden onderscheiden, kunnen in het slice model directe effecten worden gemeten in verschillende organen onder gelijke omstandigheden waarbij deze effecten het netto resultaat zijn van niet alleen binding aan de nucleaire receptor maar ook van de opname, metabolisme en uitscheiding van de liganden alsmede van de aanwezigheid van co-activatoren en repressoren. Ook werden belangrijke speciësverschillen gevonden, waarmee wordt onderstreept dat extrapolatie van gegevens uit proefdieronderzoek niet zonder meer naar de mens kunnen worden geëxtrapoleerd. *In vitro*

onderzoek met humane slices kan een belangrijke bijdrage leveren om humane gegeven te verkrijgen en het dierproefgebruik te verminderen.

Appendix

List of Abbreviations

Acknowledgments

Curriculum vitae

Awards

List of Publications

List of Abbreviations

$1,25(OH)_2D_3$	1α,25-dihydroxyvitamin D_3
ABC	ATP-binding cassette
ASBT	apical sodium dependent bile acid transporter
BAs	bile acids
BIS-I	bisindolyl maleimide I
BSEP	bile salt export pump
BUD	budesonide
C	colon
CA	cholic acid
CAR	constitutive active/androstane receptor
CDCA	chenodeoxycholic acid
CYP	cytochrome P450 enzyme
DEX	dexamethasone
DMEs	drug metabolizing enzymes
DMSO	dimethyl sulfoxide
DTs	drug transporters
DTT	dithiothreitol
EtOH	ethanol
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FXR	farnesoid X receptor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GR	glucocorticoid receptor
GRE	glucocorticoid receptor response element
HNF	hepatocyte nuclear factor
I	Ileum
J	jejunum
LCA	lithocholic acid
LRH-1	liver receptor homolog-1
LXRα	liver X receptor alpha
MeOH	methanol

MDR (P-gp)	multidrug resistance protein or P-glycoprotein
MRP	multidrug resistance-associated protein
NR	nuclear receptor
NRRE	nuclear receptor response element
NTCP	rat sodium taurocholate co-transporting polypeptide
OATP	organic anion transporting polypeptides
OST	rat organic solute transporter
PAR	pregnane activated receptor
PCS	precision-cut slices
PCIS	precision-cut intestinal slices
PCLS	precision-cut liver slices
PKC	protein kinase C
PMA	phorbol-12-myristate-13-acetate
PMSF	phenylmethylsulfonyl fluoride
PXR	pregnane X receptor
RXR	retinoid X receptor
SI	small intestine
SLC	solute carrier
SHP	short heterodimer partner
SXR	steroid X receptor
SULT2A1	sulfotransferase 2A1
UW	university of wisconsin
VDR	vitamin D receptor
WME	Williams medium E with Glutamax-I
XRE	xenobiotic response element

This thesis has more than four years and a number of people - teachers, colleagues, students' friends and acquaintances are behind it, without them it would not have been possible for me to present this work.

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Curriculum Vitae

Ansar Ali Khan was born on 3rd January 1974 in Mahabubnagar, India. He completed his senior secondary school in 1989. He studied Biochemistry as one of the major in Andhra University, Vishakapatanam, India. After successfully obtaining his Bachelor's degree in the year 1995, he joined Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), Pondicherry, India for pursuing his Masters degree in Medical Biochemistry. After obtaining his Masters degree in 1998, he joined Dr. Reddy's laboratories (Discovery Research), Hyderabad, India. He worked in the department of drug metabolism and pharmacokinetics in the capacity of senior biochemist and Research Associate. In December 2003 he joined the drug metabolism and pharmacokinetics department of Glenmark research center, Mumbai, India in the capacity of Research scientist. In February 2005 he joined the department of pharmacokinetics and drug delivery, Rijksuniversiteit, Groningen, The Netherlands, as a Ph. D student with Prof. dr. G.M.M. Groothuis as supervisor. During his Ph. D, he collaborated with various scientists at the University Medical Center Groningen and University of Toronto. The present thesis is the result of last 4 years of research in close collaboration with prof dr. Robert J. Porte, prof dr. Rutger ploeg, dr. Vincent B. Nieuwenhuijs and dr. Henri leuvenink (University Medical Center, Groningen) and Prof. dr. K. Sandy Pang (University of Toronto).

Awards

- Awarded **"Ubbo Emmius International Scholarship"** for 4 years (since February 1, 2005 to March - 2009) to pursue Ph.D at university of Groningen

- Rewarded for the contribution for the progress of **1 anti COPD molecule (GRC-3886)** into clinical trials (2004) at Glenmark Research Center, Mumbai, India.

- Rewarded for the contribution for the progress of **3 anti-diabetic molecules (DRF-2593, DRF-2725 and DRF-4158)** into clinical trials (1999 – 2004) at Dr. Reddy's Laboratories, Discovery Research, Hyderabad, India.

- Secured **3rd rank** in a entrance conducted by **JIPMER (1995),** Pondicherry, India to peruse **masters in Medical Biochemistry**.

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- **Khan AA,** Chow EC, van Loenen-Weemaes AM, Porte RJ, Pang KS and Groothuis GM (2009). Comparison of effects of VDR versus PXR, FXR and GR ligands on the regulation of CYP3A isozymes in rat and human intestine and liver. *European Journal of Pharmaceutical Sciences* 2009*;* **37**:115-125.

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