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Research Paper

LXR Alpha Transactivates Mouse Organic Solute Transporter Alpha and Beta via IR-1 Elements Shared with FXR

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Purpose. Recently identified organic solute transporter (Ost) α and β are located on the basolateral membrane of enterocytes and may be responsible for the intestinal absorption of many substrates including bile acids. In the present study, the mechanism governing the transcriptional regulation of their expression was investigated.

Methods and Results. To clarify the transcriptional regulation of Osts, reporter gene assays were performed using mouse Ostα/β promoter-luciferase reporter constructs. Co-transfection of the constructs with farnesoid X receptor (FXR) and retinoid X receptor α (RXRα) or liver X receptor α (LXRα) and RXRα into Caco-2 cells induced the transcriptional activities of both Ost α and β and further increases were observed following treatment with each agonist. Sequence analyses indicated the presence of IR-1 regions in Ostα and Ostβ promoters, which was confirmed by the finding that the deletion of IR-1 sequences abolished the response to FXR and LXRα. Furthermore, mutations in IR-1 reduced the FXR- and LXRα-dependent transactivation of Ostα/β. Together with the detection of direct binding of FXR/RXRα and LXRα/RXRα to the IR-1 elements, the presence of functional FXRE/LXRE was revealed in the promoter region of both Ostα and Ostβ. In addition, the stimulatory effect of FXR/RXRα and LXRα/RXRα on Ostα, but not on Ostβ, was further enhanced by HNF-4α.

Conclusions. It was concluded that LXRα/RXRα transcriptionally regulate mouse Ostα/β via IR-1 elements shared with FXR/RXRα. Exposure to FXR/LXRα modulators may affect the disposition of Ostα/β substrates.

KEY WORDS: bile acids; drug transporter; enterohepatic circulation; intestinal transport; transcriptional regulation.

INTRODUCTION

Recent studies revealed the presence of several kinds of drug transporters in the small intestine. For example, it is well established that P-glycoprotein (ABCB1) and breast cancer resistance protein (BCRP/ABCG2) are expressed on the apical membrane of enterocytes and are responsible for the cellular extrusion of their substrates (1–4). Concerning the apical uptake, peptide transporter 1 (PEPT1/SLC15A1) is responsible for the absorption of substrate drugs such as β-lactam antibiotics (5,6). Recently, it was suggested that organic anion transporting polypeptides (OATPs/SLC21As) play an important role in the intestinal absorption of several drugs (7–10). Moreover, the apically located ileal Na^+-dependent bile acid transporter (ASBT/SLC10A2) is responsible for the Na^+-dependent absorption of bile acids (11,12). The behavior of bile acids is also important with regard to the oral absorption of hydrophobic compounds such as cyclosporin A, since micellar formation by bile salts is important for the intestinal absorption of these drugs.

In contrast to these apical transporters, much less information is available regarding the transporters located on the basolateral membrane. Although it is possible that multidrug resistance-associated protein 3 (MRP3/ABCC3) is responsible for the absorption of anionic compounds including bile salts, it is highly expressed in the colon, but not in the ileum (13–15). Recently identified organic solute transporter α and β (Ostα/β) are the most important candidates as far as the ileal absorption of many substrates is concerned. Indeed, it has been shown that bile acids, estrone 3-sulfate, prostaglandin E_2 and digoxin are transported by Ostα/β (16,17). In particular, the demonstration of the bidirectional transport of

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ABBREVIATIONS: ASBT, apical sodium-dependent bile acid transporter; BSEP, bile salt export pump; CDCA, chenodeoxycholic acid; EMSA, electrophoretic mobility shift assay; FXR, farnesoid X receptor; FXRE, FXR/RXR binding element; HNF-4, hepatocyte nuclear factor-4; I–BABP, ileal–bile acid binding protein; IR-1, inverted repeat-1; LXR, liver X receptor; LXRE, LXR/RXR binding element; OST, organic solute transporter; RXR, retinoid X receptor.

bile acids by Ostα/β suggests the role of these transporters in the extraction of substrates from enterocytes into the blood circulation (18). The inhibitory effect of many compounds such as spironolactone, probenecid and indomethacin suggests that these transporters have broad substrate specificity.

Cumulative results prove that several metabolizing enzymes and transporters responsible for the cholesterol–bile acid homeostasis are transcriptionally regulated by nuclear receptors, particularly by a bile acid sensor, the farnesoid X receptor (FXR) and an oxysterol sensor, the liver X receptor (LXR) (19,20). Indeed, it has very recently been reported that the expression of mouse and human OSTα/β is under the control of FXR/RXRα (21–25). In the present study, we examined (1) whether LXRα/RXRα are also involved in the transcriptional regulation of mouse Ostα/β, and if so, (2) whether the mechanism is shared with the transcriptional activation by FXR/RXRα. We also examined whether HNF-4α, which is well known to be involved in lipid and bile acid homeostasis by regulating the transcription of enzymes and transporters (26, 27), further stimulates the transcriptional activation of Ostα/β mediated by the nuclear receptors.

MATERIALS AND METHODS

Materials. Restriction enzymes and Premix Taq™ were purchased from TAKARA BIO INC (Shiga, Japan). pGEM-T Easy Vector System, Dual-Luciferase Reporter Assay System, pGL3-Basic and pRL-TK vectors were purchased from Promega KK (Tokyo, Japan). QuickChange mutagenesis kits were purchased from Stratagene (La Jolla, CA). Phenol red-free Dulbecco's Modified Eagle Medium and penicillin–streptomycin were purchased from GIBCO (Tokyo, Japan). Charcoal-absorbed fetal bovine serum was purchased from Hyclone (Logan, Utah). FUGENE6 was purchased from Roche Diagnostics (Tokyo, Japan). Chenodeoxycholic acid (CDCA) was purchased from Wako (Osaka, Japan), while TO-901317 and 9-cisRA were purchased from Sigma Aldrich, Inc (St. Louis, MO).

Cloning of the 5′-flanking region of mouse Ostα/β and vector construction for reporter gene assay. The promoter region of mouse Ostα was obtained from the MouseBLAST (GenBank™ accession number AC087556). Based on the mouse Ostβ cDNA sequence, the BLAST algorithm revealed the 5′-flanking sequence of Ostβ (GenBank™ accession number AC114645). Based on the sequences obtained, Ostα promoter sense primer (5′-CTCCATCCATCTGTACCATC-3′), Ostα promoter antisense primer (5′-GTCCCTTCCACC TCTTTCAG-3′), Ostβ promoter sense primer (5′-CAACTG TCGGAACTGACCTGGTC-3′) and Ostβ promoter antisense primer (5′-TGAGAGTACTCACCCTGGAC-3′) were prepared. Amplification by these pairs of primers using genome extracted from ddY mice tails as a template generated an approximately 1.5 kb fragment of the promoter region of Ostα (from −1,454 to +88) and an approximately 0.6 kb fragment of the promoter region of Ostβ (from −527 to +40). Amplified products were inserted into pGEM-T Easy Vector and the entire sequences were verified. Sequences of the obtained 5′-flanking region of Ostβ were identical to the sequence in the GenBank™, although the amplified Ostα promoter exhibited five differences: (1) at nt −1,342, t instead of c; (2) at nt −1,182, t instead of c; (3) at nt −1,157, a instead of g; (4) absence of 12 bases (TAAATAAATAAA) originally located between nt −530 and −529 in our sequence; and (5) at nt −127, g instead of t. Direct sequencing of high fidelity PCR products confirmed these differences, implying the presence of sequence variations in the Ostα promoter among different strains of mice. Using plasmids obtained as a template, fragments obtained by PCR with the *KpnI* site attached at the 5′-end and the *Hin*dIII site attached at the 3′-end were inserted into the *KpnI*/*Hin*dIII site of the pGL3-Basic vector. The resulting plasmids were p-1454α/Luc containing the region from −1,454 to +88 of the Ostα promoter, p-645α/Luc with −645 to +88, and p-527β/Luc containing the region from −527 to +40 of the Ostβ promoter, p-141β/Luc with −141 to +40 and p-59β/Luc with −59 to +40.

Construction of Ostα/β promoter mutant. Inverted repeat-1 (IR-1) elements in the Ostα/β promoter regions were mutated by the site-directed mutagenesis technique. The sense and antisense primers listed below were used to construct the mutated vectors (Mut-p-1454α/Luc and Mut-p-527β/Luc). For Mut-p-1454α/Luc, the sense and antisense primers were 5′-GTGTGGCTGAAATCACTGATTTTCAGCAGC-3′ and 5′-GCTGCTGAAAATCAGTGATTTCAGCCACAC-3′, respectively. For Mut-p-527β/Luc, the sense and antisense primers were 5′-CATTCGTGGAATCATTCACTTTGG ACTGGC-3′ and 5′-GCCAGTCCAAAGTGAATGATTCC ACGAATG-3′, respectively.

Vector construction for the expression of nuclear receptors. LXRα and RXRα cDNA was amplified by PCR from total RNA of mouse liver. The complete LXRα cDNA was amplified with the *NheI* site attached at the 5′-end, and with the *KpnI* site attached at the 3′-end by PCR, and then inserted into pcDNA3.1(+) vector plasmid. After the amplified RXRα cDNA was inserted into pGEM-T Easy Vector, inserted fragments digested with *NotI* were ligated into pcDNA3.1(+) vector plasmid. HNF-4α amplified by PCR from total RNA of HepG2 cells with the *NotI* site attached at the 5′-end and with the *Eco*RI site attached at the 3′-end was inserted into pcDNA3.1(+) vector plasmid.

Luciferase assay. Caco-2 cells were plated on Day 0 at a density of 2.5×10^4 cells/well on 24-well plates and grown in phenol red-free Dulbecco's Modified Eagle Medium with 10% charcoal-absorbed fetal bovine serum and 1% penicillin–streptomycin. On Day 3, cells were transfected with 500 ng/well of pGL3-Basic vector, with or without Ostα/β promoter, using FUGENE6 at a DNA/lipid ratio of 1:3. In some experiments, co-transfections were performed by adding 100 ng each of human FXR and/or human RXRα in pSG5 expression vector (28), adding 100 ng each of LXRα and/or RXRα in pcDNA3.1(+) vector, or 500 ng of HNF-4α in pcDNA3.1(+) vector. All wells were also co-transfected with 50 ng of pRL-TK vector to correct the transfection efficiency. At 22 h after transfection, 100 μM CDCA or 1 μM TO-901317 was added to the culture medium and the incubation was continued for an additional 24 h. After incubation, luciferase activities were quantified by Luminescencer MCA (ATTO, Tokyo, Japan) using the Dual-Luciferase Reporter Assay System according to the manufacturer's protocol. Luciferase activities were normalized for transfection efficiency using renilla luciferase activities from pRL-TK vector. All experiments were per-

formed in triplicate and the results were confirmed by additional experiments performed at least once.

Electrophoretic Mobility Shift Assay. Electrophoretic mobility shift assays (EMSAs) were performed with in vitro translated proteins and a DIG Gel Shift Kit (Roche Diagnostics, Tokyo, Japan) according to the manufacturer's protocol. FXR, LXRα and RXRα proteins were synthesized using the TNT Quick Coupled Transcription/Translation Systems (Promega KK, Tokyo, Japan). In supershift experiments, FXR antibody (C-20, Santa Cruz Biotechnology) was added to the synthesized proteins. The sense strand sequences for Ostα and Ostβ IR-1 sequences (wt Ostα and wt Ostβ) were 5′-GTGTGGCTGAGGTCACTGACCTTCAGCAGC-3′ and 5′-CATTCGTGGGGTCATTCACCCTG GACTGGC-3′, respectively, whereas the mutated Ostα and Ostβ IR-1 sequences (mut Ostα and mut Ostβ) were 5′-GTGTGGCTGAAATCACTGATTTTCAGCAGC-3′ and 5′-CATTCGTGGAATCATTCACTTTGGACTGGC-3′, respectively. Mutations for the EMSAs were the same as those introduced in the luciferase assay.

Real-time quantitative PCR. For determination of the expression levels of endogenous OST α and β, Caco-2 cells were plated on Day 0 at a density of 5.0×10^4 cells/well on 12-well plates and grown in phenol red-free Dulbecco's Modified Eagle Medium with 10% charcoal-absorbed fetal bovine serum and 1% penicillin-streptomycin. On Day 3, cells were transfected with 200 ng each of LXRα and RXRα in pcDNA3.1(+) vector and 1 μg of HNF-4α in pcDNA3.1(+) vector using FUGENE6 at a DNA/lipid ratio of 1:3. At 22 h after transfection, 1 μM TO-901317 and 1 μM 9-cisRA were added to the culture medium and the incubation was continued for an additional 24 h. After incubation, cells were harvested with ISOGEN (Nippongene, Tokyo, Japan) and RNA obtained was reverse-transcribed with ReverTra Ace (TOYOBO, Osaka, Japan). Real-time quantitative PCR was performed using 2x SYBR GREEN (Stratagene, La Jolla, CA) and Chromo4 (BIORAD, Tokyo, Japan) with 95°C 10 min followed by the 40 cycles at 95°C for 15 s, 50°C for 30 s and 72°C for 30 s. Primers for human OSTα sense primer (5′-TGTTGGGCCCTTTCCAATAC-3′), antisense primer (5′-GAATAGGGAGGCGAACAAGC-3′), human OSTβ sense primer (5′-CAGGCAAGCAGAAAAGAAAC GATG-3′), antisense primer (5′-CCGGAAGGAAAAC TGACAGC-3′), β-actin sense primer (5′-TTCAACACCCC AGCCATGTACG-3′) and antisense primer (5′-GTGGT GGTGAAGCTGTAGCC-3′) were used.

To clarify the effect of LXR agonist on the expression of Ost α and β in vivo, 50 mg kg^{-1} day^{-1} of TO-901317 was orally administered to male C57BL/6J mice for 3 days. Tissue samples were collected with ISOGEN and RNA obtained was reverse-transcribed with ReverTra Ace. Real-time quantitative PCR was performed in the same manner with the cell experiments using these primers: mouse Ostα sense primer (5′-TACAAGAACACCCTTTGCCC-3′), antisense

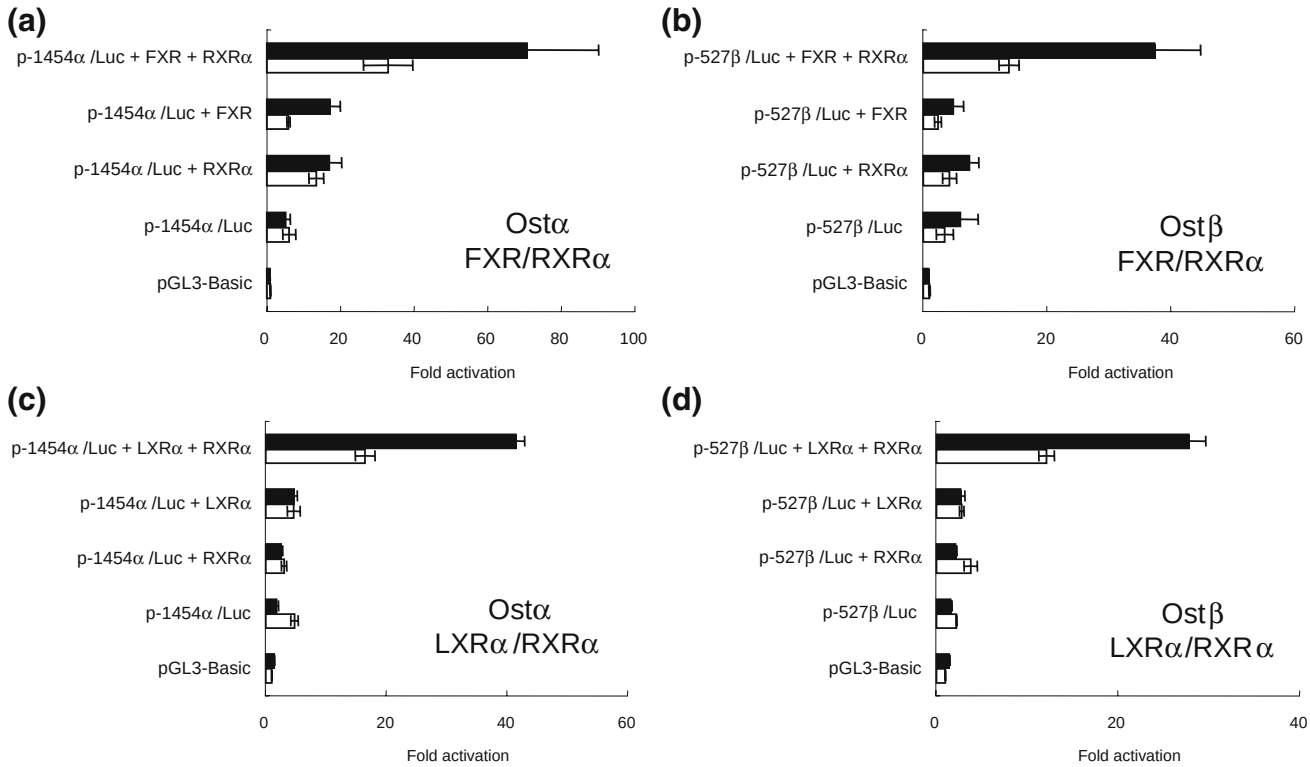


Fig. 1. Activation of reporter-linked mouse Ost**α**/**β** promoters. Caco-2 cells were transfected with luciferase-linked mouse Ost**α** (Panels (a) and (c), p-1454**α**/Luc) or Ost**β** (Panels (b) and (d), p-527**β**/Luc) promoter constructs, with or without expression vectors for FXR and/or RXR**α** (Panels (a) and (b)) or LXR**α** and/or RXR**α** (Panels (c) and (d)). Twenty-two hours later, cells were incubated for an additional 24 h in the presence or absence of 100 **μ**M CDCA (Panels (a) and (b)) or 1 **μ**M TO-901317 (Panels (c) and (d)) in order to carry out the luciferase assay. Fold activation values were calculated as the relative activities of pGL3-Basic-transfected cells in the absence of ligands. *Open* and *closed bars* represent the results obtained in the absence and presence of ligands, respectively. Values are expressed as the mean ± S.E. (n=6).

primer (5′-AGGAATCCAGAGACCAAAGC-3′), mouse Ostβ sense primer (5′-GTATTTTCGTGCAGAAGAT GCG-3′), antisense primer (5′-ATTTCTGTTTGCCAGG ATGCTC-3′), β-actin sense primer (5′-TTCAACACCCCA GCCATGTACG-3′) and antisense primer (5′-GTGGTG GTGAAGCTGTAGCC-3′).

RESULTS

FXR/RXRα and LXRα/RXRα increase the transcriptional activities of mouse Ostα/β. To determine whether mouse Ostα/β are transcriptionally regulated by FXR or LXRα, an approximately 1.5 kb fragment of Ostα promoter and a 0.6 kb fragment of Ostβ promoter were inserted upstream of the reporter luciferase gene. These constructs were transiently transfected into Caco-2 cells with FXR and/or RXRα or LXRα and/or RXRα expression vectors and were incubated in the presence or absence of each agonist (100 μM CDCA for FXR and 1 μM TO-901317 for LXRα). The luciferase activity of both Ostα and Ostβ was induced by co-transfection of FXR/RXRα or LXRα/RXRα, and a further increase in transcriptional activity was observed for both Ostα/β in the presence of each agonist (Fig. 1). These results indicate that both FXR/RXRα and LXRα/RXRα stimulate the transcription of mouse Ostα/β.

IR-1 regions upstream of Ostα/β. The increase in the reporter gene activity suggests the presence of an interaction between the Ostα/β promoter and nuclear receptors. Analyses of the 5′-flanking sequences of Ostα/β revealed the presence of IR-1 regions, consisting of two nuclear receptor half sites of the consensus sequence (AGGTGA) organized as an inverted repeat separated by a single nucleotide. These sequences were located at nt −1,229 to −1,217 in the mouse Ostα promoter (Fig. 2a) and at nt −187 to −175 in the mouse Ostβ promoter (Fig. 2b).

Deletion of IR-1 regions in the promoter constructs of Ostα/β. Reporter plasmids containing several lengths of the 5′-flanking regions were constructed for mouse Ostα and Ostβ (Fig. 3a and c, respectively). These constructs were transiently transfected into Caco-2 cells, with or without FXR/RXRα or LXRα/RXRα, and incubated in the presence or absence of each ligand. Deletion of the −1,229 to −1,217 IR-1 region clearly reduced the luciferase activity in the Ostα promoter for both FXR/RXRα and LXRα/RXRα (Fig. 3b). Similarly, in the Ostβ promoter, deletion of the −187 to −175 IR-1 region resulted in an obvious reduction in relative activity (Fig. 3d). These results suggest the involvement of the IR-1 regions in response to FXR and LXRα on the Ostα/β promoter.

Mutations of IR-1 in the Ostα/β promoter. To demonstrate directly that IR-1 regions are responsible for the transactivation of Ostα/β promoter by FXR/RXRα and LXRα/RXRα, mutations in both halves of the IR-1 element were introduced by site-directed mutagenesis (Fig. 4a and c for Ostα and Ostβ, respectively). Caco-2 cells were transfected with Ostα/β promoter-luciferase reporter constructs containing the wild type or mutated IR-1 motifs. Mutations in IR-1 abolished the response to the FXR and LXR ligands in the Ostα promoter construct (Fig. 4b). The 5′-flanking region of Ostβ showed similar results in that the mutations in IR-1 reduced the induction observed in the presence of nuclear receptors and each ligand (Fig. 4d). These observations suggest that the IR-1 regions upstream of Ostα/β act as functional FXREs and LXREs with regard to the transcriptional regulation of mouse Ostα/β.

Fig. 2. Nucleotide sequences of mouse Ost**α** and **β** promoter. The nucleotide sequences of the 5′-flanking region of mouse Ost**α** (Panel (a)) and Ost**β** (Panel (b)) inserted in p-1454**α**/Luc and p-527**β**/Luc, respectively, are shown. The numbering of the nucleotides is relative to the first nucleotide in exon 1 (*+1*, *arrow*). Note that the IR-1 elements are underlined; at −1,229 to −1,217 in the Ostα promoter and −187 to −175 in the Ostβ promoter.

FXR/RXRα and LXRα/RXRα heterodimers directly bind to IR-1 motifs in Ostα/β. EMSAs were performed to detect the direct binding of FXR/RXRα and LXRα/RXRα to the IR-1 elements in mouse Ostα and Ostβ promoter (Fig. 5). Shifted bands, which disappeared in the presence of an excess (100-fold) of unlabelled competitors, were observed with the in vitro synthesized FXR/RXRα or LXRα/RXRα heterodimers. Mutated IR-1 lost the binding capacity of nuclear receptors and could not work as a competitor against IR-1. Furthermore, the supershifted band was detected in the presence of FXR antibody in addition to FXR/RXRα. Together with the results of reporter gene assay, results

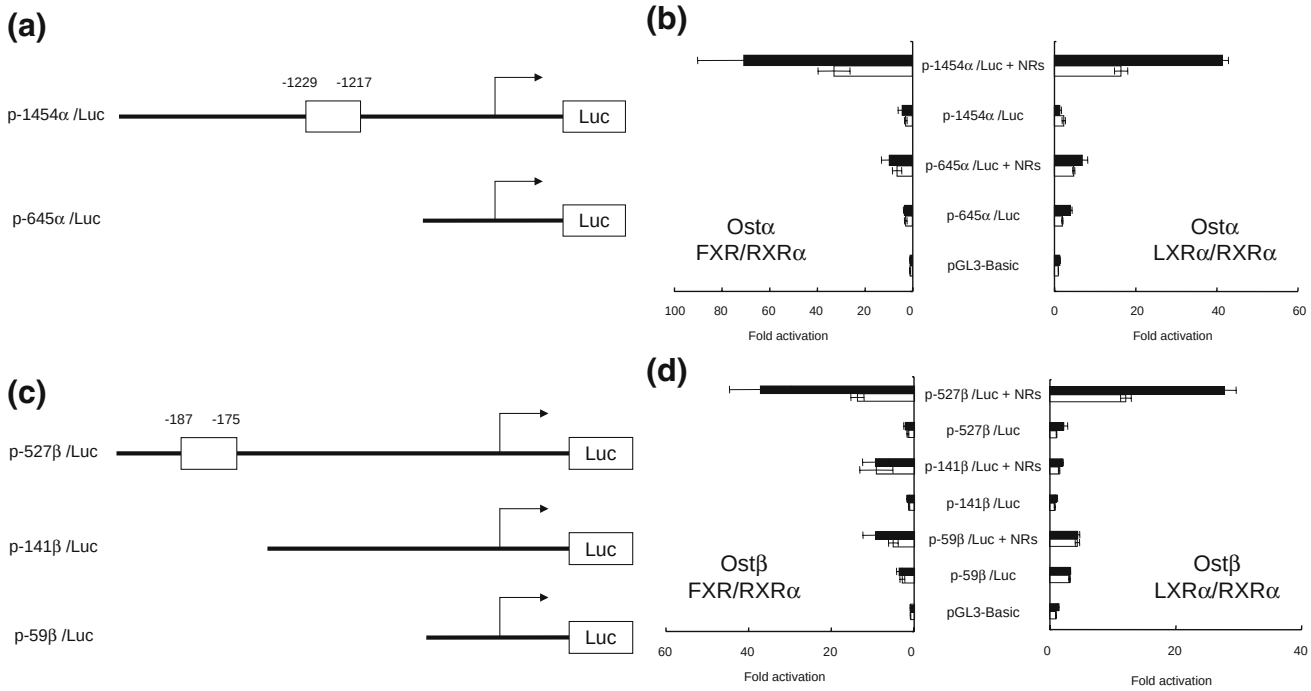
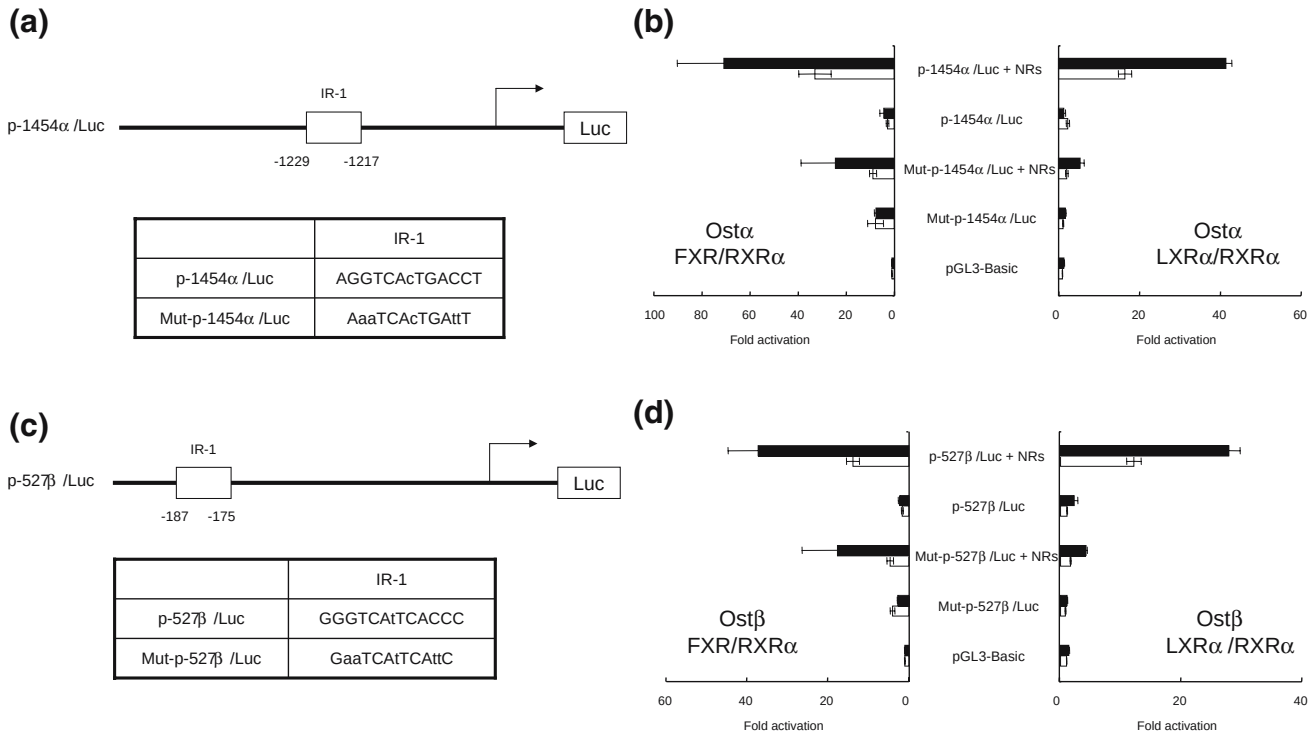


Fig. 3. Effect of deletion of IR-1 on the promoter activity. Several lengths of Ost**α** promoter (Panel (a); p-1454**α**/Luc and p-645**α**/Luc) and Ost**β** promoter (Panel (c); p-527**β**/Luc, p-141**β**/Luc and p-59**β**/Luc) were inserted into the pGL3-Basic vector. The luciferase activity of these constructs, with or without the expression vectors for nuclear receptors (NRs; FXR/RXR**α** or LXR**α**/RXR**α**), was evaluated in the presence or absence of 100 μM CDCA or 1 μM TO-901317, respectively (Panels (b) and (d)). Fold activation values were calculated as the relative activities of pGL3-Basic-transfected cells in the absence of ligands. *Open* and *closed bars* represent the results obtained in the absence and presence of ligands, respectively. Values are expressed as the mean ± S.E. (n=6).



	IR-1
p-1454α /Luc	AGGTCAcTGACCT
Mut-p-1454α /Luc	AaaTCAcTGAttT

	IR-1
p-527β /Luc	GGGTCAtTCACCC
Mut-p-527β /Luc	GaaTCAtTCAttC

Fig. 4. Effect of mutations in IR-1 on the promoter activity. Mutations were introduced into Ost**α** and Ost**β** promoter regions. The mutated sequences are given in Panels (a) and (c) for the promoter regions of Ost **α** and **β**, respectively. Caco-2 cells were transfected with wild type or mutant of luciferase-linked mouse Ost**α** (Panel (b)) or Ost**β** (Panel (d)) promoter constructs in order to perform the luciferase assay. Fold activation values were calculated as the relative activities of pGL3-Basic-transfected cells in the absence of ligands. *Open* and *closed bars* represent the results obtained in the absence and presence of ligands (100 μM CDCA for FXR/RXRα or 1 μM TO-901317 for LXRα/RXRα), respectively. Values are expressed as the mean ± S.E. (n=6).

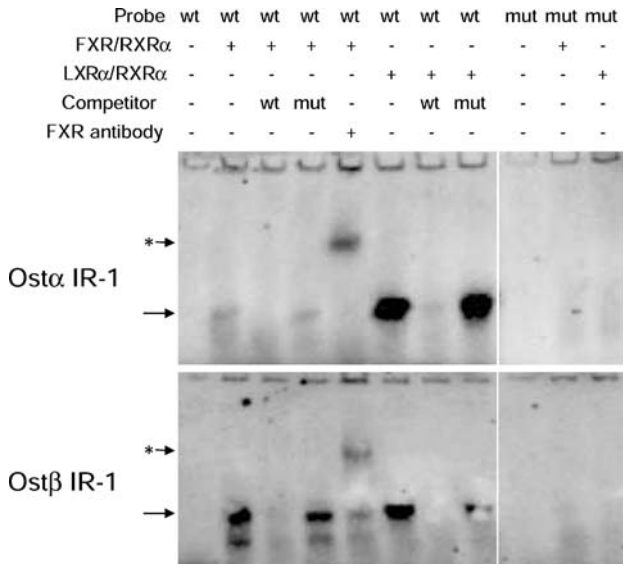


Fig. 5. Binding of FXR/RXR**α** and LXR**α**/RXR**α** on IR-1 sequences in mouse Ost **α** and **β** promoters. Eletctrophoretic mobility shift assays (EMSAs) were performed for the IR-1 element in Ost**α** and Ost**β** promoter. The *arrows* indicate the positions of complex containing the DNA probe and nuclear receptors and *asterisks with arrowheads* denote the positions of the supershifted bands. Competitor analysis was performed with 100-fold excess of unlabeled IR-1 or mutated IR-1 sequences.

obtained in EMSAs reveal the presence of functional FXRE/LXRE in Ostα (−1,229 to −1,217) and Ostβ (−187− to −175) promoters.

HNF-4α stimulates the transcriptional activities of mouse Ostα. To investigate the effect of HNF-4α on the transactivation of Ostα/β by FXR and LXRα, luciferase assays were carried out using mouse Ostα (p-1454α/Luc) and Ostβ (p-527β/Luc) promoter constructs. Transactivation of Ostα by FXR/RXRα and LXRα/RXRα was dramatically stimulated by the co-transfection of HNF-4α, whereas transcriptional activities of Ostβ showed little alteration in the presence of HNF-4α (Fig. 6). Results of the real-time quantitative PCR appeared to be consistent with these observations (Fig. 7). Although induction of endogenous OSTα seemed to be stimulated by the transfection of HNF-4α, little stimulatory effect was observed on OSTβ.

LXR agonist treatment induces Ostα and Ostβ in vivo. To clarify the involvement of LXR-dependent regulation in vivo, mice were treated with TO-901317. Increased expression of both Ostα and Ostβ was observed in the liver and intestine, although the increase was not statistically significant in the intestine. These results suggest the in vivo relevance of LXR pathway on the transcriptional regulation of Ostα/β (Fig. 8).

DISCUSSION

In the present study, analyses using the 5′-flanking region of mouse Ostα/β demonstrated that both FXR and LXRα are involved in the transactivation of both Ostα and Ostβ. Together with the detection of the direct binding of FXR/RXRα and LXRα/RXRα heterodimers to the IR-1 elements, Ostα/β are revealed to be transcriptionally regulated by the functional FXREs/LXREs in Ostα (−1,229 to −1,217) and Ostβ (−187 to −175) promoters. Although it was very recently suggested that Ostα/β are transactivated by FXR (21–25), we were able to demonstrate that LXRα is also involved in the regulation of Ostα/β by the mechanism shared with FXR. In addition, we could demonstrate that HNF-4α is also involved in the transcriptional regulation of Ostα mediated by FXR and LXRα.

Ostα and Ostβ proteins are reported to be localized on the basolateral membrane of enterocytes (18). Based on the fact that the exogenous expression of Ostα or Ostβ alone did not result in the functional expression of each Ost and that, in contrast, simultaneous transfection of both Ostα/β resulted in the acquisition of significant transport activity for their substrates (16–18), it is assumed that Ostα/β act as a heterodimer. The results obtained in the present study demonstrate the coordinate regulation of both mouse Ost α and β by FXR/RXRα and LXRα/RXRα via functional FXREs/LXREs in their promoter region. Although direct interaction between Ost α and β proteins has not been confirmed at present, coordinate regulation of Ostα/β by their transport substrate is consistent with the hypothesis that Ost

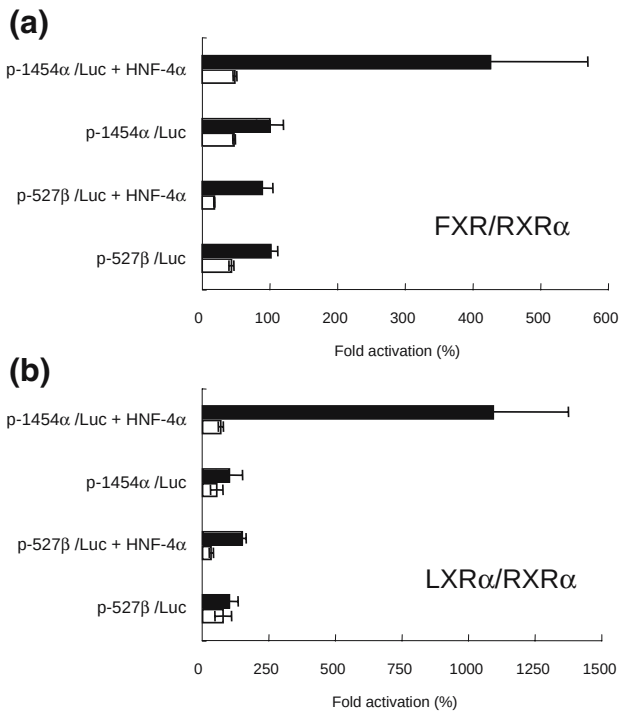


Fig. 6. Stimulation of transcriptional activities of mouse Ost**α** and Ost**β** by HNF-4**α**. Caco-2 cells were transfected with luciferase-linked mouse Ost**α** (p-1454**α**/Luc) or Ost**β** (p-527**β**/Luc) promoter constructs with expression vectors for FXR/RXR**α** and HNF-4**α** (Panel A) or LXR**α**/RXR**α** and HNF-4**α** (Panel B). Twenty-two hours later, cells were incubated for an additional 24 h in the presence or absence of 100 μM CDCA (Panel (a)) or 1 μM TO-901317 (Panel (b)) in order to carry out the luciferase assay. Fold activation values were calculated as a percentage of relative activities compared with that of p-1454**α**/Luc or p-527**β**/Luc in the presence of ligands. *Open* and *closed bars* represent the results obtained in the absence and presence of ligands, respectively. Values are expressed as the mean ± S.E. (n=6).

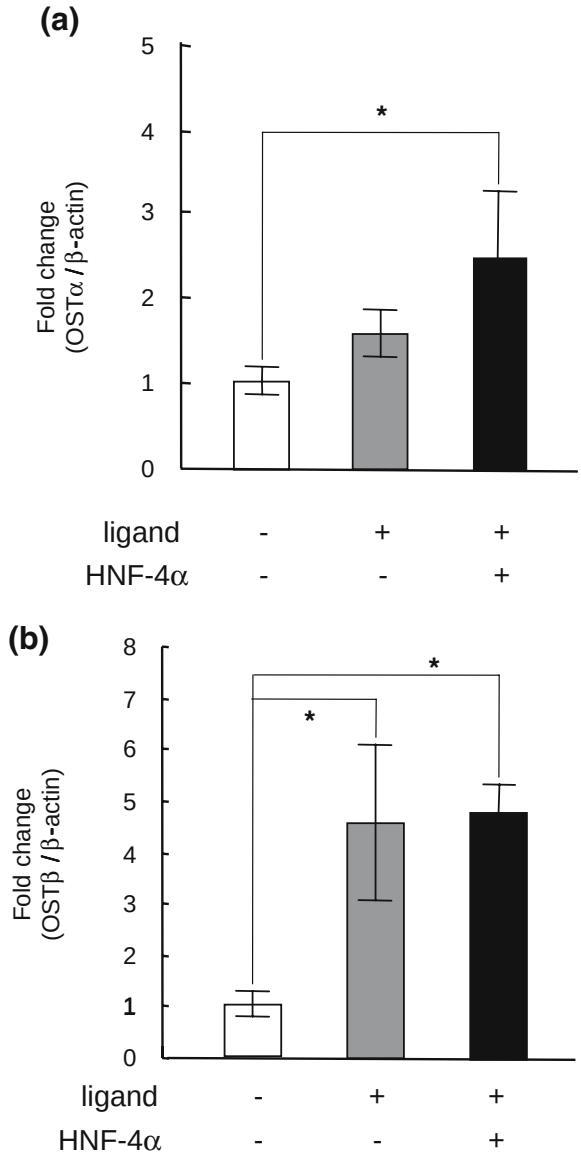


Fig. 7. Effect of LXR**α** and HNF-4**α** on the endogenous expression of OST**α** and OST**β** in Caco-2 cells. Caco-2 cells were transfected with the expression vectors for LXR**α**, RXR**α** and HNF-4**α**. Twenty-two hours later, cells were incubated for an additional 24 h in the presence or absence of ligands (1 μM TO-901317 and 1 μM 9-cisRA). RNA obtained from cells were analyzed by real-time PCR as described in "Materials and Methods". Optical density of the PCR products of OST**α**/**β** was normalized by the expression level of **β**-actin. Fold change values were calculated as the relative expression level compared with that of the cells in the absence of ligands and HNF-4**α**. Values are expressed as the mean ± S.E. (n=3). *Significantly different from the control cells by ANOVA followed by Dunnett test (p<0.05).

α and β should be the mutual physiological heterodimer partners.

The results of the present study have some significant implications. In a recent report, guggulsterone from the guggul tree *Commiphora mukul* appears to be a natural phytochemical which acts as an FXR antagonist (29), whereas xanthohumol, the principal prenylated chalcone from beer hops, was shown to be a ligand of FXR (30). These findings suggest the possible effect of natural products, in addition to bile acids and oxysterols, on the transcription of Ostα/β via modulation of FXR and LXRα activity. In patients prescribed drugs transported by Ostα/β, the dosage might have to be adjusted depending on the expression level of Ostα/β transporters.

Transcriptional regulation via FXR/RXRα has also important implications particularly as far as the intestinal reabsorption of bile acids is concerned. Expression of Asbt, a major bile acid importer in enterocytes, is repressed through the FXR/short heterodimer partner (SHP)/functional liver receptor homolog-1 (LRH-1) cascade in mice (31). If the concentration of bile acids in the ileum increased, enterocytes may protect themselves from the toxic effect of bile acids by controlling the expression level of Asbt and Ostα/β. Transcriptional down-regulation of Asbt reduces the cellular uptake of bile acids and induced expression of Ostα/β stimulates the efflux of bile acids to the blood side, so that the intracellular concentrations of bile acids remain constant.

The crucial roles of FXR in regulating genes encoding bile acid transporters are also supported by studies in FXR-

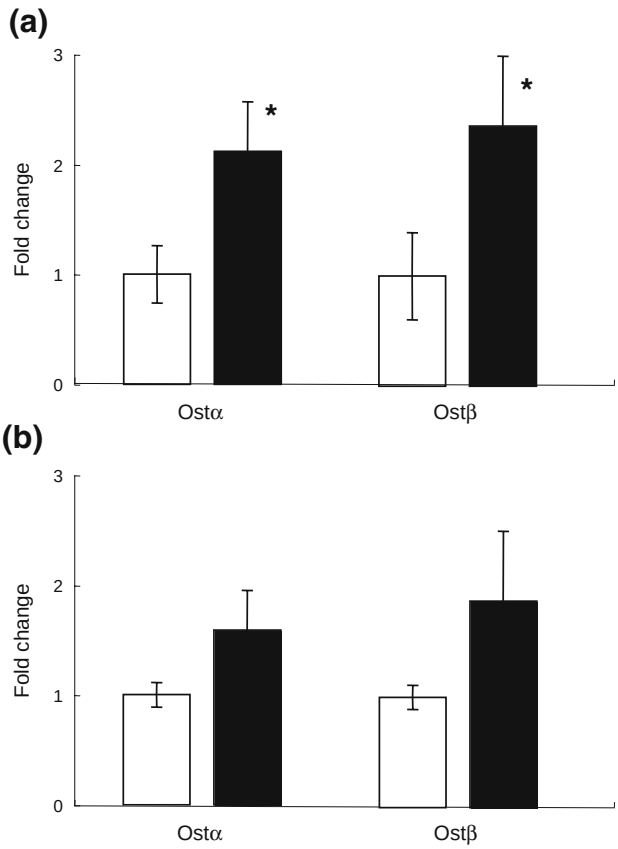


Fig. 8. Effect of LXR agonist on the expression of Ost**α** and Ost**β** in the mouse liver and intestine. RNA obtained from the liver (Panel (a)) and intestine (Panel (b)) of TO-901317-treated and control mice were analyzed by real-time PCR as described in "Materials and Methods". Optical density of the PCR products of Ost**α**/**β** was normalized by the expression level of **β**-actin. Fold change values were calculated as the relative expression level compared with that of control mice. *Open* and *closed bars* represent the results obtained in the absence and presence of treatment with TO-901317, respectively. Values are expressed as the mean ± S.E. (n=4). *Significantly different from the control mice by Student's t-test (p<0.05).

deficient mice, which have reduced bile salt export pump (Bsep/Abcb11) expression in the liver and undetectable ileal–bile acid binding protein (I–BABP) expression in the small intestine as well as exhibiting a lack of inducible expression of both genes following administration of a diet rich in cholic acid (32). In addition, it was recently demonstrated that the feeding of FXR agonists resulted in an increase in the expression level of intestinal Ostα/β in mice (24,25) and this induction disappeared in FXR-null mice (25). Furthermore, *ex vivo* experiments showed enhanced expression of OSTα/β by CDCA in the human ileum (23). Although no information is available on the expression of Ostα/β in LXR-null mice, it was indicated that the oral administration of LXR agonist led to the induction of Ostα/β in the liver and intestine (Fig. 8).

In the present study, it was demonstrated that Ostα/β are under the transcriptional regulation of LXRα/RXRα, although the physiological significance of the regulation remains unclear. We would like to discuss the transcriptional regulation of ASBT and I–BABP, which are also thought to be involved in the intestinal absorption of bile acids. It is reported that the down-regulation of ASBT is caused not only by FXR agonists but also by cholesterol (33) and, in addition, I–BABP is induced by LXRα/RXRα as well as by FXR/RXRα (34). It is thus suggested that ASBT, I–BABP and Ostα/β, all of which should be involved in the intestinal transport of bile acids, are regulated by bile acids and sterols. The positive regulation of Ostα/β and I–BABP by FXR/RXRα and LXRα/RXRα may be related to the unidentified cooperative function of these two proteins in the intestinal absorption of bile acids.

We could also demonstrate that LXRα/RXRα share the common binding element with FXR/RXRα. As the binding element of FXR/RXRα and LXRα/RXRα, we could identify the IR-1 elements in both Ost α and β promoter. Although it is well established that IR-1 acts as a common FXRE, only a limited number of reports are available which suggest that IR-1 also acts as a LXRE; for only I–BABP and phospholipid transfer protein, the IR-1 elements were identified as the FXRE/LXRE (34,35). Our results of LXRα/RXRα-dependent induction of human OSTα/β in Caco-2 cells suggest the possible function of FXREs as LXREs in human OSTα promoter (GGGTGAaTGACCT and AGGCCAgTGA CCC) and OSTβ promoter (AGGTCAgTCACCC) (Fig. 7) (23,24).

Furthermore, we could also demonstrate that the transcriptional stimulation of Ostα promoter by FXR/RXRα and LXRα/RXRα is further enhanced by HNF-4α, while HNF-4α did not enhance the transcriptional stimulation of Ostβ (Fig. 6). These results may be accounted for by considering the fact that mouse Ostα, but not Ostβ, has some putative HNF-4α binding sites in its proximal promoter region, although we cannot exclude the possibility of indirect effect of HNF-4α, such as HNF-4α-mediated activation of other transcriptional factors. This is the first demonstration that HNF-4α stimulates the transcriptional activation by FXR/RXRα and LXRα/RXRα. Recently, the direct protein–protein interaction and transcriptional cooperation of pregnane X receptor and HNF-4α were reported on the cytochrome P-450 (CYP) 3A4 promoter (36). A similar mechanism may exist for FXR and LXRα and the difference in the affinity of interaction may explain the different stimulatory effect of HNF-4α on FXR/RXRα and LXRα/RXRα (Fig. 6).

Collectively, the results of the present study show that both mouse Ostα and Ostβ, which form the heterodimer to transport many substrates including bile acids, are the direct targets of LXRα/RXRα via IR-1 elements on their promoters shared with FXR/RXRα. Furthermore, the regulation of Ostα gene by these nuclear receptors was enhanced by HNF-4α. It is also possible that this transport system is affected by FXR and LXRα agonists/antagonists, which are present in a number of natural products.

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REFERENCES

1. H. Suzuki and Y. Sugiyama. Role of metabolic enzymes and efflux transporters in the absorption of drugs from the small intestine. *Eur. J. Pharm. Sci.* **12**:3–12 (2000).
2. H. Kusuhara and Y. Sugiyama. Role of transporters in the tissue-selective distribution and elimination of drugs: transporters in the liver, small intestine, brain and kidney. *J. Control. Release* **78**:43–54 (2002).
3. C. G. Dietrich, A. Geier, and R. P. Oude Elferink. ABC of oral bioavailability: transporters as gatekeepers in the gut. *Gut* **52**:1788–1795 (2003).
4. Q. Mao and J. D. Unadkat. Role of the breast cancer resistance protein (ABCG2) in drug transport. *Aaps J.* **7**:E118–E133 (2005).
5. T. Okano, K. Inui, H. Maegawa, M. Takano, and R. Hori. H+ coupled uphill transport of aminocephalosporins via the dipeptide transport system in rabbit intestinal brush-border membranes. *J. Biol. Chem.* **261**:14130–14134 (1986).
6. M. E. Ganapathy, M. Brandsch, P. D. Prasad, V. Ganapathy, and F. H. Leibach. Differential recognition of beta -lactam antibiotics by intestinal and renal peptide transporters, PEPT 1 and PEPT 2. *J. Biol. Chem.* **270**:25672–25677 (1995).
7. D. Kobayashi, T. Nozawa, K. Imai, J. Nezu, A. Tsuji, and I. Tamai. Involvement of human organic anion transporting polypeptide OATP-B (SLC21A9) in pH-dependent transport across intestinal apical membrane. *J. Pharmacol. Exp. Ther.* **306**:703–708 (2003).
8. H. Satoh, F. Yamashita, M. Tsujimoto, H. Murakami, N. Koyabu, H. Ohtani, and Y. Sawada. Citrus juices inhibit the function of human organic anion-transporting polypeptide OATP-B. *Drug Metab. Dispos.* **33**:518–523 (2005).
9. G. K. Dresser, D. G. Bailey, B. F. Leake, U. I. Schwarz, P. A. Dawson, D. J. Freeman, and R. B. Kim. Fruit juices inhibit organic anion transporting polypeptide-mediated drug uptake to decrease the oral availability of fexofenadine. *Clin. Pharmacol. Ther.* **71**:11–20 (2002).
10. G. K. Dresser, R. B. Kim, and D. G. Bailey. Effect of grapefruit juice volume on the reduction of fexofenadine bioavailability: possible role of organic anion transporting polypeptides. *Clin. Pharmacol. Ther.* **77**:170–177 (2005).
11. A. L. Craddock, M. W. Love, R. W. Daniel, L. C. Kirby, H. C. Walters, M. H. Wong, and P. A. Dawson. Expression and transport properties of the human ileal and renal sodium-dependent bile acid transporter. *Am. J. Physiol.* **274**:G157–G169 (1998).
12. M. Kida, Y. Mano, Y. Ueno, K. Kobayashi, J. Goto, M. Ishii, and T. Shimosegawa. Vectorial transport of bile acids in immortalized mouse bile duct cells. *Hepatol. Res.* **27**:151–157 (2003).

13. T. Hirohashi, H. Suzuki, H. Takikawa, and Y. Sugiyama. ATP-dependent transport of bile salts by rat multidrug resistance-associated protein 3 (Mrp3). *J. Biol. Chem.* **275**:2905–2910 (2000).
14. D. Rost, S. Mahner, Y. Sugiyama, and W. Stremmel. Expression and localization of the multidrug resistance-associated protein 3 in rat small and large intestine. *Am. J. Physiol.: Gastrointest. Liver Physiol.* **282**:G720–G726 (2002).
15. T. Shoji, H. Suzuki, H. Kusuhara, Y. Watanabe, S. Sakamoto, and Y. Sugiyama. ATP-dependent transport of organic anions into isolated basolateral membrane vesicles from rat intestine. *Am. J. Physiol.: Gastrointest. Liver Physiol.* **287**:G749–G756 (2004).
16. W. Wang, D. J. Seward, L. Li, J. L. Boyer, and N. Ballatori. Expression cloning of two genes that together mediate organic solute and steroid transport in the liver of a marine vertebrate. *Proc. Natl. Acad. Sci. USA* **98**:9431–9436 (2001).
17. D. J. Seward, A. S. Koh, J. L. Boyer, and N. Ballatori. Functional complementation between a novel mammalian polygenic transport complex and an evolutionarily ancient organic solute transporter, OSTalpha-OSTbeta. *J. Biol. Chem.* **278**:27473–27482 (2003).
18. P. A. Dawson, M. Hubbert, J. Haywood, A. L. Craddock, N. Zerangue, W. V. Christian, and N. Ballatori. The heteromeric organic solute transporter alpha-beta, Ostalpha-Ostbeta, is an ileal basolateral bile acid transporter. *J. Biol. Chem.* **280**:6960–6968 (2005).
19. R. G. Tirona and R. B. Kim. Nuclear receptors and drug disposition gene regulation. *J. Pharm. Sci.* **94**:1169–1186 (2005).
20. M. Makishima. Nuclear receptors as targets for drug development: regulation of cholesterol and bile acid metabolism by nuclear receptors. *J. Pharmacol. Sci.* **97**:177–183 (2005).
21. J. L. Boyer, M. Trauner, A. Mennone, C. J. Soroka, S. Y. Cai, T. Moustafa, G. Zollner, J. Y. Lee, and N. Ballatori. Upregulation of a basolateral FXR-dependent bile acid efflux transporter OSTalpha-OSTbeta in cholestasis in humans and rodents. *Am. J. Physiol.: Gastrointest. Liver Physiol.* **290**:G1124–G1130 (2006).
22. T. Frankenberg, A. Rao, F. Chen, J. Haywood, B. L. Shneider, and P. A. Dawson. Regulation of the mouse organic solute transporter alpha-beta, Ostalpha-Ostbeta, by bile acids. *Am. J. Physiol.: Gastrointest. Liver Physiol.* **290**:G912–G922 (2006).
23. J. F. Landrier, J. J. Eloranta, S. R. Vavricka, and G. A. Kullak-Ublick. The nuclear receptor for bile acids, FXR, transactivates human organic solute transporter-alpha and -beta genes. *Am. J. Physiol.: Gastrointest. Liver Physiol.* **290**:G476–G485 (2006).
24. H. Lee, Y. Zhang, F. Y. Lee, S. F. Nelson, F. J. Gonzalez, and P. A. Edwards. FXR regulates organic solute transporters alpha and beta in the adrenal gland, kidney, and intestine. *J. Lipid Res.* **47**:201–214 (2006).
25. G. Zollner, M. Wagner, T. Moustafa, P. Fickert, D. Silbert, J. Gumhold, A. Fuchsbichler, E. Halilbasic, H. Denk, H. U. Marschall, and M. Trauner. Coordinated induction of bile acid detoxification and alternative elimination in mice: role of FXR-regulated organic solute transporter-alpha/beta in the adaptive response to bile acids. *Am. J. Physiol.: Gastrointest. Liver Physiol.* **290**:G923–G932 (2006).
26. M. Crestani, E. De Fabiani, D. Caruso, N. Mitro, F. Gilardi, A. B. Vigil Chacon, R. Patelli, C. Godio, and G. Galli. LXR (liver X receptor) and HNF-4 (hepatocyte nuclear factor-4): key regulators in reverse cholesterol transport. *Biochem. Soc. Trans.* **32**:92–96 (2004).
27. D. Jung, B. Hagenbuch, M. Fried, P. J. Meier, and G. A. Kullak-Ublick. Role of liver-enriched transcription factors and nuclear receptors in regulating the human, mouse, and rat NTCP gene. *Am. J. Physiol.: Gastrointest. Liver Physiol.* **286**:G752–G761 (2004).
28. J. Grober, I. Zaghini, H. Fujii, S. A. Jones, S. A. Kliewer, T. M. Willson, T. Ono, and P. Besnard. Identification of a bile acid-responsive element in the human ileal bile acid-binding protein gene. Involvement of the farnesoid X receptor/9-cis-retinoic acid receptor heterodimer. *J. Biol. Chem.* **274**:29749–29754 (1999).
29. J. Cui, L. Huang, A. Zhao, J. L. Lew, J. Yu, S. Sahoo, P. T. Meinke, I. Royo, F. Pelaez, and S. D. Wright. Guggulsterone is a farnesoid X receptor antagonist in coactivator association assays but acts to enhance transcription of bile salt export pump. *J. Biol. Chem.* **278**:10214–10220 (2003).
30. H. Nozawa. Xanthohumol, the chalcone from beer hops (Humulus lupulus L.), is the ligand for farnesoid X receptor and ameliorates lipid and glucose metabolism in KK-A(y) mice. *Biochem. Biophys. Res. Commun.* **336**:754–761 (2005).
31. F. Chen, L. Ma, P. A. Dawson, C. J. Sinal, E. Sehayek, F. J. Gonzalez, J. Breslow, M. Ananthanarayanan, and B. L. Shneider. Liver receptor homologue-1 mediates species- and cell line-specific bile acid-dependent negative feedback regulation of the apical sodium-dependent bile acid transporter. *J. Biol. Chem.* **278**:19909–19916 (2003).
32. C. J. Sinal, M. Tohkin, M. Miyata, J. M. Ward, G. Lambert, and F. J. Gonzalez. Targeted disruption of the nuclear receptor FXR/BAR impairs bile acid and lipid homeostasis. *Cell* **102**:731–744 (2000).
33. C. Thomas, J. F. Landrier, D. Gaillard, J. Grober, M. C. Monnot, A. Athias, and P. Besnard. Cholesterol dependent downregulation of mouse and human apical sodium dependent bile acid transporter (ASBT) gene expression: molecular mechanism and physiological consequences. *Gut* **55**:1321–1331 (2006).
34. J. F. Landrier, J. Grober, J. Demydchuk, and P. Besnard. FXRE can function as an LXRE in the promoter of human ileal bile acid-binding protein (I–BABP) gene. *FEBS Lett.* **553**:299–303 (2003).
35. P. A. Mak, H. R. Kast-Woelbern, A. M. Anisfeld, and P. A. Edwards. Identification of PLTP as an LXR target gene and apoE as an FXR target gene reveals overlapping targets for the two nuclear receptors. *J. Lipid Res.* **43**:2037–2041 (2002).
36. T. Li and J. Y. Chiang. Rifampicin induction of CYP3A4 requires pregnane X receptor cross talk with hepatocyte nuclear factor 4alpha and coactivators, and suppression of small heterodimer partner gene expression. *Drug Metab. Dispos.* **34**:756–764 (2006).