Protective Effects of 6-Ethyl Chenodeoxycholic Acid, a Farnesoid X Receptor Ligand, in Estrogen-Induced Cholestasis

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Received October 26, 2004; accepted January 7, 2005

ABSTRACT

The farnesoid X receptor (FXR), an endogenous sensor for bile acids, regulates a program of genes involved in bile acid biosynthesis, conjugation, and transport. Cholestatic liver diseases are a group of immunologically and genetically mediated disorders in which accumulation of endogenous bile acids plays a role in the disease progression and symptoms. Here, we describe the effect of 6-ethyl chenodeoxycholic acid (6-ECDCA or INT-747), a semisynthetic bile acid derivative and potent FXR ligand, in a model of cholestasis induced by 5-day administration of 17α-ethynylestradiol (E217α) to rats. The exposure of rat hepatocytes to 1 μM 6-ECDCA caused a 3- to 5-fold induction of small heterodimer partner (Shp) and bile salt export pump (bsep) mRNA and 70 to 80% reduction of cholesterol 7α-hydroxylase (cyp7a1), oxysterol 12β-hydroxylase (cyp8b1), and Na+/taurocholate cotransporting peptide (ntcp). In vivo administration of 6-ECDCA protects against cholestasis induced by E217α. Thus, 6-ECDCA reverted bile flow impairment induced by E217α, reduced secretion of cholic acid and deoxycholic acid, but increased muricholic acid and chenodeoxycholic acid secretion. In vivo administration of 6-ECDCA increased liver expression of Shp, bsep, multidrug resistance-associated protein-2, and multidrug resistance protein-2, whereas it reduced cyp7a1 and cyp8b1 and ntcp mRNA. These changes were reproduced by GW4064, a synthetic FXR ligand. In conclusion, by demonstrating that 6-ECDCA protects against E217α cholestasis, our data support the notion that development of potent FXR ligands might represent a new approach for the treatment of cholestatic disorders.

Cholestasis results in systemic and intrahepatic retention of potentially toxic bile acids that causes liver injury, ultimately leading to biliary fibrosis and cirrhosis (Trauner et al., 1996). Estrogens are well known to cause intrahepatic cholestasis in susceptible women during pregnancy, administration of oral contraceptives, and postmenopausal replacement therapy (Vore, 1987; Bossard et al., 1993; Huang et al., 2000). In addition, estrogen-induced cholestasis associates with a reduced activity of the Na+/taurocholate cotransporting polypeptide (ntcp) (Bossard et al., 1993; Simon et al., 1996) and a reduction of endogenous bile acid synthesis (Simon et al., 1996).

This study was partially supported by a research grant from Intercept Pharmaceuticals (New York, NY).

Article, publication date, and citation information can be found at http://jpet.aspetjournals.org. doi:10.1124/jpet.104.079665.

ABBREVIATIONS: bsep, bile salt export pump; mdr, multidrug resistance protein; mrp, multidrug resistance-associated protein; ntcp, Na+/taurocholate cotransporting peptide; SHP, small heterodimer partner; FXR, farnesoid X receptor; cyp7a1, cholesterol 7α-hydroxylase; cyp8b1, oxysterol 12β-hydroxylase; CDCA, chenodeoxycholic acid; 6-ECDCA, 6-ethyl chenodeoxycholic acid; E217α, 17α-ethynylestradiol; qRT-PCR, qualitative reverse transcription-polymerase chain reaction; ALP, alkaline phosphatase; oapt, organic anion transporting polypeptide; β-MCA, β-muricholic acid; CA, cholic acid; LCA, lithocholic acid; TDCA, taurodeoxycholic acid; GW4064, 3-(2,6-dichlorophenyl)-4-(3’carboxy-2-chlorostilben-4-yl)-oxymethyl-5-isopropyl-isoxazole.
Although physiological actions of estrogens are mediated by the classic signaling pathway in which an estrogen receptor binds to an estrogen response element in the promoter of a gene, recent studies (Lai et al., 2003) have demonstrated that estrogens increase the liver expression of the short heterodimer partner (Shp), an atypical nuclear receptor that lacks a DNA-binding domain and is a target for farnesoid X receptor (FXR) ligands. Shp is known to repress the activity of many nuclear hormone receptors in vitro and is critically involved in regulating bile acid synthesis. Whether Shp activation mediates changes in bile acid synthesis in a rodent model of cholestasis induced by estrogen administration is still unclear.

Bile acids act as signaling molecules that regulate their own biosynthesis and transport by binding to and activating the FXR (Forman et al., 1995; Makishima et al., 1999; Parks et al., 1999; Seol et al., 1995; Wang et al., 1999, 2002). FXR alters the transcription of target genes by binding DNA sequences composed of two inverted repeats separated by one nucleotide as a heterodimer with the 9-cis-retinoic acid receptor α (Forman et al., 1995; Seol et al., 1995). Activation of FXR initiates the transcription of a cohort of genes that function to decrease the concentration of bile acids within the hepatocyte. Specifically, FXR induces the expression of the genes encoding BSEP (Sinal et al., 2000; Ananthanarayanan et al., 2001), MDR3 (Kerr et al., 2002; Wang et al., 2002; Holt et al., 2003), and MRP2. In addition, FXR activation leads to a feedback repression of cyp7a1 and cyp8b1 (Goodwin et al., 2000; del Castillo-Olivares and Gill, 2001). These genes encode for the cholesterol 7α-hydroxylase and the sterol 12α-hydroxylase, both of which are central to the synthesis of bile acids from cholesterol (Goodwin et al., 2000; del Castillo-Olivares and Gill, 2001). In rodents, the FXR-dependent suppression of cyp7a1 is mediated by Shp (Goodwin et al., 2000; del Castillo-Olivares and Gill, 2001), which interacts with liver receptor homolog-1, a known positive regulator of cyp7a1 and represses its transcriptional activity (Goodwin et al., 2000; Lu et al., 2000; del Castillo-Olivares and Gill, 2001; Kerr et al., 2002; Wang et al., 2002).

We have previously reported that modification of CDCA by addition of an ethyl group in its position 6 results in a semisynthetic bile acid, the 6-ECDCA, which has potent FXR agonist activity (Pellicciari et al., 2002; Costantino et al., 2003; Mi et al., 2003; Fiorucci et al., 2004). The resolution of the crystal structure of the FXR ligand binding domain complexed with 6-ECDCA and the coactivator peptide Src-1 (Mi et al., 2003) demonstrates that in the presence of 6-ECDCA, helix 12, the activation function 2 domain of the receptor, adopts the agonist conformation and stabilizes the binding of the coactivator peptide (Costantino et al., 2003). This would provide a mechanism for enhanced binding of coactivators through intermolecular contacts between their LXXLL sequences, explaining the enhanced potency of 6-ECDCA in comparison with the natural ligand.

Because activation of FXR might have the potential to protect the liver in cholestatic diseases (Liu et al., 2003), we have designed a study to investigate whether administration of 6-ECDCA protects against cholestasis development induced by estrogen and to investigate the molecular targets involved in this effect.

Materials and Methods

Materials. The synthesis of 6-ECDCA has been described previously (Pellicciari et al., 2002). CDCA and 17α-ethynylestradiol (E217a) were from Sigma-Aldrich (St. Louis, MO).

In Vitro Studies: Effect of 6-ECDCA on Rat FXR-Regulated Genes. Rat hepatocytes were prepared as described previously by in situ collagenase perfusion through the hepatic portal vein (Fiorucci et al., 2002). After isolation, cells were suspended in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 1 nM insulin, 0.15 mg/ml methionine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Cell viability was >96% (trypan blue dye exclusion test). Hepatocytes were then plated in matrix/Matrixgel-coated culture plates at a density of 1 × 105 cells per dish in 95% air and 5% CO2. Cells were then incubated with 1 μM 6-ECDCA for 24 h, and total RNA was extracted for quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis.

In Vivo Studies. Adult male Wistar rats weighing 300 to 350 g were used throughout the study. Before the experiments, the animals were maintained on standard chow and water ad libitum and housed in a temperature (21–23°C)- and humidity (45–50%)-controlled room under a 12-h light/dark cycle. All studies were approved by the Animal Study Committee of the University of Perugia.

Surgical Procedures and Bile Flow Measurement. For bile flow measurement, animals were anesthetized with a single dose of sodium pentobarbital (50 mg/kg body wt intraperitoneally) and maintained under this condition throughout the experiment. After catheterization of the jugular vein and carotid artery using a PE-50 polyethylene tubing (Intramedic; Clay Adams, Parsippany, NJ), a middle abdominal incision was made, and the common bile duct was also cannulated (PE-10, Intramedic; Clay Adams). Body temperature was maintained at 37.0 to 38.5°C with a warming lamp to prevent hypothemic alterations of bile flow. Bile flow was determined by gravimetry, assuming a density of the bile of 1.0 g/ml. The biliary excretion rate was calculated as the product between bile flow and biliary concentration. The bile was assayed for both total and individual bile salts by high-pressure liquid chromatography as described previously (Setchell et al., 1997). Total bile acid concentrations were determined by the sum of individual bile acids (Russell and Setchell, 1992).

Estrogen-Induced Cholestasis. Animals were randomly divided into experimental groups (8–12 rats/group) as described in Table 1. For bile collection, surgical procedures were made on the sixth day (i.e., 1 day after the administration of the last dose of E217a). Bile collection started between 9:00 and 11:00 AM to minimize influence of circadian variations. Bile was collected at 15-min intervals for 120 min, and bile flow was determined gravimetrically (Kern et al., 1977; Steiger et al., 1994; Koopen et al., 1999). At the end of the study, the livers were perfused for 30 s with saline, and the major lobe was removed. Liver samples were gently frozen in liquid nitrogen and preserved at −80°C until used for qRT-PCR studies.

Routine Serum Biochemistry and Bile Acid Measurements. Serum samples were stored at −70°C until analysis of aspartate aminotransferase, bilirubin, alkaline phosphatase (ALP), and γ-glutamyl transpeptidase by routine clinical chemistry testing performed on a Hitachi 717 automatic analyzer.

Table 1

<table>
<thead>
<tr>
<th>Treatment Regimen</th>
<th>No. of Rats</th>
<th>Duration of Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12</td>
<td>5 days</td>
</tr>
<tr>
<td>E217a, 5 mg/kg</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>E217a + 6-ECDCA, 1 mg/kg</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>E217a + 6-ECDCA, 5 mg/kg</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>E217a + 6-ECDCA, 10 mg/kg</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>E217a + CDCA, 10 mg/kg</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>E217a + GW4064, 30 mg/kg</td>
<td>8</td>
<td>5</td>
</tr>
</tbody>
</table>
qRT-PCR. After the rats had been killed, livers were removed and immediately snap-frozen on liquid nitrogen and stored at −80°C until use. Total RNA was isolated using TRIzol reagent (Invitrogen, Milan, Italy) as described previously (Fiorucci et al., 2004). Quantification of the expression rat genes was performed by qRT-PCR using the following sense and antisense primers: ntcp, 5'-gcatgatgcactc-tcatttact-3' and 5'-tcataaagggaggaatggc-3'; bsep, 5'-aagggaa-gcaagagaacttac-3' and 5'-ttactccagttgcaacaacggt-3'; cyp7a1, 5'-ctgc-gccaaagctgctt-3' and 5'-ctgtgc-ggtgtgctttggact-3'; cyp8b1, 5'-cccttatac-3' and 5'-gacattagtgaaaagattct-3'; Sdhb, 5'-cctggagcagccctcgt-3' and 5'-gaccataaggaggacaaaggtct-3'; FXR, 5'-ctggctacataagcaacacaga-3' and 5'-gtgtacacagattaggtcagtt-3'; mphp3, 5'-tcgaagctatatctgaagctcaggttattt and 5'-atgtagaagctctgattctca-3'; ntcp, 5'-gcat-gatgcactcattatc-3' and 5'-tcataaagggaggaatggc-3'; and Gapdh, 5'-tctgcctacctgcaagtg-3' and 5'-gacaggagcaatctactgta-3'. All PCR primers were designed using software PRIMER3-OUTPUT using published sequence data from the National Center for Biotechnology Information database. The RNA was reverse-transcribed with Superscript III (Invitrogen) in 20-μl reaction volume using random primers. For RT-PCR, 100 ng of template was used in 25 μl containing 0.3 μM of each primer and 12.5 μl of 2× SYBR Green PCR Master mix (Bio-Rad, Hercules, CA). All reactions were performed in triplicate, and the thermal cycling conditions were as follows: 2 min at 95°C, followed by 50 cycles of 95°C for 10 s and 60°C for 30 s in an iCycler IQ instrument (Bio-Rad). The conditions were as follows: 2 min at 95°C, followed by 50 cycles of 95°C for 10 s and 60°C for 30 s in an iCycler IQ instrument (Bio-Rad). The mean value of the replicates for each sample was calculated and expressed as the cycle threshold (CT, cycle number at which each PCR reaction reaches a predetermined fluorescence threshold, set within the linear range of all reactions). The amount of gene expression was then calculated as the difference (ΔCT) between the C_{T} value of the sample for the target gene and the mean C_{T} value of that sample for the endogenous control (Gapdh). Each gene is expressed as a ratio between control (untreated) and treated rats.

**Statistical Analysis.** Data reported are the mean ± S.E. of the number of experiments indicated. The statistical analysis was carried out using a GraphPad Prism 3 (GraphPad Software Inc., San Diego, CA). Analysis of variance and Student’s t test for paired data were used when appropriate.

**Results**

6-ECDCA Transactivates FXR and Modulates FXR-Regulated Genes in Hepatocytes. To examine whether 6-ECDCA has the ability to regulate genes involved in bile acid synthesis and transport, rat hepatocytes were incubated with a maximally effective concentration of 6-ECDCA (1 μM) and its parent molecule CDCA (100 μM) for 24 h, and mRNA expression of Shp, cyp7a1, cyp8b1, bsep, ntcp, and oatp1 was measured by qRT-PCR. As shown Fig. 1B, we found that exposure to FXR ligands increases Shp and bsep mRNA expression by 3- to 5-fold, whereas it reduced cyp7a1, cyp8b1, and ntcp mRNA expression by 50 to 70% (n = 5; P < 0.05 versus control cells). In contrast, 6-ECDCA and CDCA failed to modulate oatp1 gene expression (Fig. 1B). To investigate the effect of estrogen on the gene involved in bile acid synthesis and transport, rat hepatocytes were exposed to 100 nM E_217α. As shown in Fig. 1C, exposure to E_217α increased Shp mRNA expression by ~2-fold (n = 5; P < 0.05 versus control cells), but it failed to inhibit cyp7a1 and cyp8b1 and ntcp mRNA expression. Thus, in contrast to FXR ligands, induction of liver expression of Shp by E_217α does not translate into inhibition of cyp7a1 and cyp8b1.

**6-ECDCA Protects against Cholestasis Induced by E_217α.** Five-day administration of E_217α slightly decreased body weight (Fig. 2A), which was paralleled by increased levels of serum ALP, a biochemical marker of cholestasis (n = 6–8 animals; P < 0.05 versus control rats) and bile acids. In contrast, E_217α treatment failed to modify either ALT, bilirubin, or γ-glutamyl transpeptidase levels (not shown). Administration of 6-ECDCA (Fig. 2A–D) failed to reverse the body weight loss caused by estrogen but improved serum ALP activity (n = 6–8; P < 0.05 versus E_217α at 10 mg/kg/day 6-ECDCA). Treating rats with CDCA did not improve either the body weight, serum ALP or bile acid levels (n = 6–8; P > 0.05 versus E_217α). Consistent with these biochemical changes, bile flow analysis carried out 24 h after the last dose of 6-ECDCA or CDCA (Fig. 3, A–C; Table 2) showed an impairment of bile flow that dropped from 68.0 ± 3.4 μl/kg/min in control rats to 36.9 ± 3.5 μl/kg/min (−48 ± 5%) in rats treated with 5 mg/kg E_217α (n = 6–8; P < 0.01). Administration of 6-ECDCA induced a dose-dependent improvement of cholestatic changes caused by E_217α. At the dose 10 mg/kg/day (Fig. 3A; Table 2), it completely reverted cholestasis induced by E_217α (n = 6–8; P < 0.01 versus control rats).

![Fig. 1](https://example.com/figure1.png) 6-ECDCA is a potent and selective activator of FXR. A, chemical structure of 6-ECDCA. B, 6-ECDCA increases the expression of FXR-regulated genes in rat hepatocytes. Cells were incubated with 6-ECDCA (1 μM) for 24 h, and gene expression was monitored by qRT-PCR. Data are mean ± S.E. of four experiments. *, P < 0.05 versus untreated cells. C, exposure of rat hepatocytes to E_217α increases Shp mRNA but fails to repress cyp7a1 and cyp8b1 mRNA expression. Data are mean ± S.E. of four experiments. *, P < 0.05 versus untreated cells.
E17α). In contrast, no protection was observed in rats treated with 10 mg/kg/day CDCA (Fig. 3B; Table 2). Analysis of total bile acid output demonstrated that the decrease in total bile salt output induced by E17α was primarily accounted for by a decrease in the excretion of taurocholic acid (−55% versus control) and TDCA (−71% versus control), and to a lesser extent, β-MCA (−13% versus control) (Table 2). Administration of 6-ECDCA partially prevented the impairment in total bile acid output caused by E17α by increasing the relative abundance of β-MCA (not significantly different from animals receiving E17α alone) and TCDCA (+27%; P < 0.05 versus E17α) and TDCA (+74%; P < 0.05 versus E17α). Despite the significant enrichment in TCDCA and TDCA caused by CDCA, it failed to protect against bile flow impairment caused by E17α (n = 6–8; P > 0.05 versus E17α).

Confirming the protective role of FXR ligands on this animal model, we found that GW4064 (Fig. 1) was also effective in reducing cholestasis as measured by serum ALP activity (n = 8–10; P < 0.05 versus E17α). GW4064 (Fig. 3C; Table 2) also attenuated bile flow impairment caused by E17α (n = 8–10; P < 0.05 versus E17α) and increased the relative abundance of β-MCA (+13%; P > 0.05 versus E17α) and TCDCA (+55%; P < 0.05 versus E17α) and TDCA (+28%; P > 0.05 versus E17α).

As shown in Fig. 4, 5-day administration of E17α slightly increased liver Shp mRNA expression and reduced cyp7a1 and cyp8b1 mRNA by 40 to 70% (n = 4; P < 0.01 versus control) (Fig. 4, A–C). Furthermore, E17α treatment decreased ntcp and oatp1 mRNA expression by ~30 to 40% (n = 4; P < 0.01 versus control; Fig. 5, A and D), but it had no effect on bsep mRNA levels (n = 4; P > 0.05 versus control; Fig. 5G). Furthermore, E17α administration was associated with ~20% reduction of mrp2 mRNA (Fig. 5B), whereas it caused an ~8-fold increase in mrp3 gene expression (Fig. 5E), and 2- to 4-fold induction of mdr1a and mdr1b (n = 4; P < 0.05 versus control; Fig. 5, C and F). Administration of 6-ECDCA resulted in a 3- to 4-fold increase of Shp mRNA expression (n = 4; P < 0.05 versus control; Fig. 4C), which associates with a further decrease in cyp7a1 (n = 4; P < 0.01 versus control; Fig. 4A) and cyp8b1 (n = 4; P < 0.05 versus rats administered E17α; Fig. 4B) mRNA, the latter being reduced to almost undetectable levels. Consistent with Shp induction, treating rats with 6-ECDCA caused an ~80% reduction of ntcp mRNA (n = 4; P < 0.05 versus control; Fig. 5A) and increased bsep, mrp2, and mdr2 mRNA by 2- to 4-fold; n = 4; P < 0.05 versus control; Figs. 5, B, E, and H). Administering rats with 6-ECDCA reduced mdr1a mRNA expression, whereas it had no effect on induction of mrp3 and mdr1b caused by E17β (Fig. 5C). In contrast to 6-ECDCA, CDCA failed to up-regulate Shp mRNA expression over the effect of E17α (n = 4; P < 0.05 in comparison with control), resulting in a comparable inhibition of cyp7a1 and cyp8b1 mRNA expression in the liver (n = 4; P < 0.05 versus 6-ECDCA; Fig. 5). CDCA was also less effective than its derivative in regulating ntcp, bsep, mrp2, and mdr2 mRNA (n = 4; P > 0.05 versus E17α). As shown in Fig. 5, similarly to 6-ECDCA, CDCA had no effect on either mrp3, mdr1a, or mdr1b mRNA compared with E17α (n = 4; P > 0.05).

Confirming the protective role of FXR against cholestasis development, treating rats with GW4064 increased Shp expression by ~4-fold and reduced liver cyp7a1 and cyp8b1 mRNA by 60 to 70% (P < 0.05 versus E17α). GW4064 administration increased bsep, mrp2, and mdr2 mRNA expression by 2- to 4-fold (n = 4; P < 0.05 versus control), whereas similarly to 6-ECDCA it had no effect on mrp3, mdr1a, and mdr1b (P > 0.05 versus E17α). Finally, none of the treatments were effective in modulating oatp1 mRNA (n = 4; P > 0.05 versus E17α).
TABLE 2
Effect of natural and synthetic FXR ligands on bile acid output in E217α-induced cholestasis
Numbers in parentheses indicate the percentage of each individual bile acid. Data are mean ± S.D. of 8 to 12 rats/group.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>E217α, 5 mg/kg/day i.p.</th>
<th>E217α + 6-EDCA, 10 mg/kg/day i.p.</th>
<th>E217α + CDCA, 10 mg/kg/day i.p.</th>
<th>E217α + GW4064, 30 mg/kg/day i.p.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bile flow (µl/kg/min)</td>
<td>68.0 ± 6.0</td>
<td>33.3 ± 3.0*</td>
<td>66.0 ± 4.1**</td>
<td>39.0 ± 5.0*</td>
<td>71.0 ± 4.1**</td>
</tr>
<tr>
<td>Total bile acid output (µg/kg min)</td>
<td>187.5 ± 31.2</td>
<td>129.7 ± 35.3*</td>
<td>152.6 ± 28.3**</td>
<td>298.5 ± 239.9**</td>
<td>161.3 ± 17.3**</td>
</tr>
<tr>
<td>Individual bile salts</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tauro-MCA</td>
<td>87.1 ± 11.6 (46.5%)</td>
<td>75.9 ± 16.4 (58.8%)</td>
<td>76.1 ± 14.1 (49.6%)</td>
<td>70.7 ± 54.9 (23.5%)</td>
<td>91.3 ± 10.8 (56.6%)</td>
</tr>
<tr>
<td>Taurocholic acid</td>
<td>77.1 ± 14.0 (41.1%)</td>
<td>42.3 ± 13.3* (32.0%)</td>
<td>33.1 ± 5.7* (23.7%)</td>
<td>63.0 ± 55.6 (21.3%)</td>
<td>47.0 ± 8.2 (29.1%)</td>
</tr>
<tr>
<td>TCDCDA</td>
<td>8.7 ± 2.3 (4.6%)</td>
<td>7.3 ± 3.3 (5.6%)</td>
<td>9.3 ± 0.9 (6.4%)</td>
<td>101.1 ± 92.1* (34.2%)</td>
<td>14.1 ± 1.4 (8.7%)</td>
</tr>
<tr>
<td>TDCA</td>
<td>14.7 ± 3.3 (7.8%)</td>
<td>4.2 ± 2.3* (3.4%)</td>
<td>7.6 ± 1.7* (4.9%)</td>
<td>63.7 ± 37.3* (21.1%)</td>
<td>7.9 ± 1.4 (5.6%)</td>
</tr>
<tr>
<td>Tauro-6-EDCA</td>
<td>N.D.</td>
<td>N.D.</td>
<td>26.5 ± 5.9* (17.4%)</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.D., not detected.
* P < 0.05 versus control; ** P < 0.05 versus E217α.

Fig. 3. A–C, effects of 5-day administration of 6-EDCA, CDCA, and GW4064 on bile flow in administered E217α. Bile flow was measured as described under Materials and Methods after 5 days of administration of E217α alone or in combination with 6-EDCA, CDCA, or GW4064. Data are mean ± S.E. of six to eight rats per group. *, P < 0.05 versus control; **, P < 0.05 versus E217α alone.
In vitro studies in human and rodent hepatocytes have provided evidence that FXR regulates bile acid synthesis by modulating the activity of CYP7A1, the rate-limiting enzyme in the pathway involved in conversion of cholesterol into bile acids (Chiang, 2002). In the normal liver, liver X receptor (LXR), a nuclear receptor for oxysterols, acts as a cholesterol sensor and up-regulates the expression of CYP7A1 in response to increased ligands (Goodwin et al., 2000; Lu et al., 2000). CYP7A1's enzymatic activity leads to the production of bile acids that bind to FXR and activate SHP (Peet et al., 1988; Goodwin et al., 2000; Lu et al., 2000). This regulatory circuit is supported by the finding that FXR-null mice have reduced levels of Shp and lack the ability to feedback repress cyp7a1 in response to cholic acid (CA) feeding (Sinal et al., 2000). We now demonstrated that this pathway operates also during cholestasis. Indeed, 5-day administration of E217α was associated with increased expression of Shp (Lai et al., 2003) and significant reduction (∼40%) of cyp7a1 mRNA together with a complete inhibition of cyp8b1 mRNA expression.

In vitro exposure of rat hepatocytes to estrogen increases Shp mRNA, but, in contrast to the FXR ligands, induction of Shp caused by E217 failed to repress cyp7a1 mRNA expression, suggesting that estrogen does not directly regulate this gene expression (Lai et al., 2003). The basis for the lack of repression of cyp7a1 and cyp8b1 despite the induction of Shp by estrogen in rodents remains unclear. In SHP-deficient mice, CA still represses cyp7a1 expression via alternative mechanisms, suggesting that SHP induction by itself might not be sufficient to mediate repression of cyp7a1 and cyp8b1 without additional signaling pathways (Goodwin et al., 2000; Lu et al., 2000). FXR activation could provide additional signals such as production of an SHP ligand with the ability to confer SHP repression upon CYP7A1 and CYP8B1 promoters.

E217α administration is associated with a number of changes in the expression of basolateral and canalicular transporters (Fig. 6A). Thus, 5-day administration of E217α caused an ∼30 to 40% reduction of ntcp and oatp1 mRNA (Stieger et al., 1994; Trauner et al., 1998; Huang et al., 2000; Kullak-Ublick et al., 2003). Because ntcp is an important component of the sinusoidal systems that import bile acid into hepatocytes, and its expression is regulated by FXR-SHP regulatory cascade (Kullak-Ublick et al., 2003), a reduction of this transporter is likely to develop as an adaptation to represent cholestasis.

Our qRT-PCR analysis also demonstrates that the levels of mrp3, mdr1a, and mdr1b were increased in rats administered with E217α. These canalicular transporters mediate efflux of xenobiotics and basolateral excretion of anion conjugates from hepatocytes. mdr1a and mdr1b expression is regulated by pregnane X receptor (Maglich et al., 2002; Goodwin et al., 2003). Since pregnane X receptor also is activated, among other ligands, by bile acids (Kast et al., 2002; Goodwin et al., 2003), induction of mdr1a and mdr1b is likely to represent an adaptive change that protects the liver by providing a mechanism for clearance of toxic constituents from hepatocytes.

Bile acids bind and activate FXR, with CDCA being the most active ligand (Forman et al., 1995; Makishima et al., 1999; Parks et al., 1999; Wang et al., 1999). Activation of FXR by endogenous ligand, however, requires micromolar concentrations, suggesting that development of potent FXR agonists, active in the nanomolar range, could help to activate this sensor mechanism by providing a mechanism for clearance of toxic constituents from hepatocytes. One potential risk of FXR agonists, however, might result from the feedback inhibition of bile acid synthesis, a mechanism that might cause a further impairment of cholestasis by reducing the biliary flow driven by bile salts. Our results, however, demonstrate that administration of potent FXR ligands protects against cholestasis development despite that it completely inhibited cyp7a1 and cyp8b1 mRNA expression. The
The main explanation for this effect is that although CYP7A1 catalyzes the key, rate-limiting step of the classical, major pathway of the overall bile salt synthesis, bile acids can also be generated through an alternative pathway that involves the mitochondrial sterol 27-hydroxylase (CYP27A1) (Russell and Setchell, 1992). In contrast to the classical pathway that leads to synthesis of CA and deoxycholic acid (DCA), CYP27A1 catalyzes the first step in the alternative pathway, which produces selectively CDCA and the bile salts derived from its further hepatic and/or intestinal conversion, i.e., MCA (Russell and Setchell, 1992). CYP27A1 is not regulated by FXR, suggesting that bile acid synthesis through this pathway could be maintained even in the presence of FXR ligands. Consistent with this view, the analysis of individual bile acids in the bile of E\textsubscript{17α}treated rats (Table 2) demonstrated a relative enrichment of the bile salt pool by tauro-β-MCA and TCDCA (i.e., bile acids generated through the alternative pathway) together with a decrease of CA and its secondary bile salt deoxycholic acid (Kern et al., 1977; Stieger et al., 1994; Koopen et al., 1999).

In the present study, we have demonstrated that a synthetic FXR ligand protects against development of cholestasis induced by estrogen administration to rats. At the dose of 10 mg/kg, 6-ECDCA profoundly inhibited ntcp expression and caused, along with a 3-fold increase of Shp, a 2- to 3-fold increase in bsep and mrp2 and a 5-fold increase in mdr2 mRNA. Since these basolateral (ntcp) and canalicular (bsep, mrp2, and mdr2) transporters are directly regulated by FXR (Denson et al., 2001) and their repression/induction is not observed or is only partially observed (ntcp) in rats treated with E\textsubscript{17α} alone, their regulation is likely to play a mechanistic role in the anticholestatic effect of 6-ECDCA (Kullak-Ublick et al., 2003). Supporting this view, it has been shown that the cis- and trans-inhibition of bsep is the main determinant of impaired bile flow in this model (Kullak-Ublick et al., 2003). BSEP and MRP2 are ATP-binding cassette proteins that transport bile acids and other organic anions across the canalicular membrane (Kullak-Ublick et al., 2003). Canalicular transport of bile acids is a rate-limiting step in hepatic excretion of bile acids and bsep and mrp2 are critically involved in this process. Mutation of BSEP and MRP2 genes in humans associated with cholestatic disorders (Trauner et al., 1998; Kullak-Ublick et al., 2003). Thus, inactivating mutation of BSEP gene give rise to the type 2 progressive familial intrahepatic cholestasis (Trautnicks et al., 1998), whereas mutations of MRP2 give rise to Dubin-Johnson syndrome (Paulusma et al., 1997), a disease characterized by high serum bilirubin due to the inability of liver to excrete this metabolite into the bile. Induction of BSEP and MRP2 expression is under the control of FXR, and their modulation in response to CA feeding is lost in FXR null mice.
(Sinal et al., 2000). Similarly to BSEP, MRP2 has an inverted repeat-1-responsive element in the promoter region (Kullak-Ublick et al., 2004) that directly binds the FXR/retinoid X receptor heterodimer and is activated by CDCA and 6-ECDCA in vitro. The demonstration that bsep expression is unchanged in rats rendered cholestatic by estrogen administration but is induced by 6-ECDCA and GW4064, however, indicates that both positive and negative mediators are generated in the liver of cholestatic animals that might affect the expression of this canalicular transporter. Interestingly, the cholestatic bile acid lithocholic acid (LCA) inhibits the BSEP promoter and strongly decreases BSEP expression induced by CDCA in human hepatocytes through antagonism of FXR activity (Yu et al., 2002). Although LCA is a weak FXR agonist it might function as a partial antagonist in vivo, displacing more potent endogenous ligands from their binding site. In contrast to CDCA, however, bsep induction caused by 6-ECDCA cannot be reverted in vitro by LCA (S. Fiorucci, unpublished data), suggesting that potent FXR ligands might overcome the effect of LCA in vivo.

In addition to bsep and mrp2, we have shown that 6-ECDCA administration increases mdr2/MDR3 expression by ∼5-fold. Induction of mdr2/MDR3 by the FXR ligand GW4064 has recently been described in cholestasis induced by bile duct ligation and GW4064 has recently been described in cholestasis induced by bile duct ligation and GW4064, however, indicates that both positive and negative mediators are generated in the liver of cholestatic animals that might affect the expression of this canalicular transporter. Interestingly, the cholestatic bile acid lithocholic acid (LCA) inhibits the BSEP promoter and strongly decreases BSEP expression induced by CDCA in human hepatocytes through antagonism of FXR activity (Yu et al., 2002). Although LCA is a weak FXR agonist it might function as a partial antagonist in vivo, displacing more potent endogenous ligands from their binding site. In contrast to CDCA, however, bsep induction caused by 6-ECDCA cannot be reverted in vitro by LCA (S. Fiorucci, unpublished data), suggesting that potent FXR ligands might overcome the effect of LCA in vivo.

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Since 6-ECDCA is a CDFCA derivative, there is a possibility that its effects are due to its conversion into CDAO. However, this seems not to be the case. Indeed, we found that T-CDCA concentrations did not increased in rats treated with 6-ECDCA, whereas a significant enrichment was observed in animals treated with CDAO alone, suggesting that 6-ECDCA is not converted to CDAO during its liver metabolism.

In conclusion, we have provided evidence that induction of the canalicular transporters bsep, mrp2, and mdr2/MDR3 and repression of bile acid biosynthesis mediate anticholestatic effects of 6-ECDCA. By demonstrating that 6-ECDCA protects against cholestasis induced in rat by estrogen administration, our data support the notion that development of potent FXR agonists might be beneficial in the treatment of cholestatic disorders.

References


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