Liver Cirrhosis
the Antifibrotic Activity of FXR Ligands in Rodent Models of Hepatic Fibrosis

ABSTRACT

The nuclear receptors farnesoid X receptor (FXR) and peroxisome proliferator-activated receptor $\gamma$ (PPAR$\gamma$) exert counter-regulatory effects on hepatic stellate cells (HSCs) and protect against liver fibrosis development in rodents. Here, we investigated whether FXR ligands regulate PPAR$\gamma$ expression in HSCs and models of liver fibrosis induced in rats by porcine serum and carbon tetrachloride administration and bile duct ligation. Our results demonstrate that HSCs trans-differentiation associated with suppression of PPAR$\gamma$ mRNA expression, whereas FXR mRNA was unchanged. Exposure of cells to natural and synthetic ligands of FXR, including 6-ethyl chenodeoxycholic acid (6-ECDCA), a synthetic derivative of chenodeoxycholic acid, reversed this effect and increased PPAR$\gamma$ mRNA by $\approx$40-fold. Submaximally effective concentrations of FXR and PPAR$\gamma$ ligands were additive in inhibiting $\alpha(1)$ collagen mRNA accumulation induced by transforming growth factor (TGF)$\beta_1$. Administration of 6-ECDCA in rats rendered cirrhotic by porcine serum and carbon tetrachloride administration or bile duct ligation reverted down-regulation of PPAR$\gamma$ mRNA expression in HSCs. Cotreatment with 6-ECDCA potentiates the antifibrotic activity of rosiglitazone, a PPAR$\gamma$ ligand, in the porcine serum model as measured by morphometric analysis of liver collagen content, hydroxyproline, and liver expression of $\alpha(1)$ collagen, $\alpha$-smooth muscle actin, fibronectin, TGF$\beta_1$, and tissue inhibitor of metalloproteinase 1 and 2, whereas it enhanced the expression of PPAR$\gamma$ and uncoupling protein 2, a PPAR$\gamma$-regulated gene, by 2-fold. In conclusion, by using an in vitro and in vivo approach, we demonstrated that FXR ligands up-regulate PPAR$\gamma$ mRNA in HSCs and in rodent models of liver fibrosis. A FXR-PPAR$\gamma$ cascade exerts counter-regulatory effects in HSCs activation.

Hepatic fibrosis is a scarring process of the liver that includes both increased and altered deposition of extracellular matrix components (Friedman, 2003). In chronic liver disease, hepatic stellate cells (HSCs) undergo a process of trans-differentiation (Friedman, 2003) from a resting, fat-storing, phenotype toward a myofibroblast-like phenotype characterized by expression of fibroblastic cell markers such as $\alpha(1)$ collagen and $\alpha$-smooth muscle actin ($\alpha$-SMA). Although the mediators involved in this process are not completely understood, a growing body of evidence suggests that members of the nuclear receptor (NR) superfamily (Fiorucci et al., 2004b) exert counter-regulatory effects acting as braking signals to prevent HSCs trans-differentiation.

The farnesoid X receptor (FXR) is a ligand-activated transcription factor that regulates cholesterol and fatty acid metabolism and functions as an endogenous sensor for bile acids (Forman et al., 1995; Makishima et al., 1999; Parks et al.,...
1999; Wang et al., 1999). FXR alters the transcription of target genes by binding as a heterodimer with the retinoid X receptor to response elements (FXR response elements) consisting of an inverted repeat of the canonical AGGTCA hexanucleotide core motif spaced by 1 base pair (Forman et al., 1995). We have recently shown that in addition to its ability to modulate bile acid synthesis and excretion, FXR functions as a negative regulator of α1-collagen (I) synthesis in HSCs and attenuates/reverses fibrosis in rodent models of liver fibrosis (Fiorucci et al., 2004b). Activation of FXR in HSCs leads to induction of the short heterodimer partner (SHP) (Goodwin et al., 2000) that counteracts HSCs activation induced by transforming growth factor (TGF)β1 and thrombin (Fiorucci et al., 2004b).

Peroxisome proliferator-activated receptors (PPARs) are a family of ligand-activated NR that, similarly to FXR, bind regulatory element in responsive genes after the formation of a heterodimeric complex with retinoid X receptor α (Berger and Moller, 2002). Three mammalian PPAR subtypes have been identified: PPARα, β (or δ), and γ (Berger and Moller, 2002). PPARβ and γ have been found in rat and human HSCs (Galli et al., 2000; Marra et al., 2000; Miyahara et al., 2000; Hellemans et al., 2003). However, although PPARγ ligands (Galli et al., 2002; Kon et al., 2002) inhibit proliferation, migration, and chemokine expression of HSCs and protect against development of liver fibrosis, induction of PPARβ in HSCs favors the development of an activated phenotype (Hellemans et al., 2003).

Previous studies have provided evidence that FXR ligands increase PPARα mRNA expression in human hepatocytes (Pineda Torra et al., 2003). Whether FXR interacts with PPARγ, however, is unknown. In the present study, we demonstrate that natural and synthetic FXR ligands induce PPARγ expression in HSCs and provide evidence that a FXR ligand protects against PPARγ down-regulation caused by liver diseases and enhances the antifibrotic activity of PPARγ ligands. These results provide the first molecular evidence for a cross-talk between the FXR and PPARγ and suggest that NRs provide a network of counter-regulatory signals that limit HSCs activation.

Materials and Methods

Porcine serum, Sirius red, fast green, CCl4, ursodeoxycholic acid were from Sigma-Aldrich (St. Louis, MO). 6-α-Ethyl-chenodeoxycholic acid (6-ECDCA) was synthesized as described previously (Pellecchiari et al., 2002; Mi et al., 2003). GW4064 (Maloney et al., 2000) was kindly donated by Tim Wilson (GlaxoSmithKline, Research Triangle Park, NC). GW9662 was from Alexis Biochemicals (Florence, Italy).

In Vitro Studies

Isolation and Culture of HSCs. In vitro studies were performed on primary cultures of rat HSCs and HSC-T6, a rat immortalized HSC line. Primary rat HSCs were isolated from control and cirrhotic rats according to techniques described previously (Fiorucci et al., 2004a,b). The HSCs were more than 90% viable as assessed by trypan blue exclusion and >95% pure. Cells were cultured at 37°C in an atmosphere of 5% CO2 in Dulbecco’s modified minimal essential medium (Invitrogen, Carlsbad, CA) containing 1% fetal calf serum (FCS), 2 mM L-glutamine, and 5000 IU/ml penicillin/5000 g/ml streptomycin.

To investigate the expression of FXR and PPARα, β, and γ in HSCs, and the effect of FXR and PPARγ ligands on HSCs activation, primary culture of rat HSCs (days 0 and 7) and 24-h starved HSC-T6 cells were incubated for 18 h with medium alone or increasing concentrations of 6-ECDCA, a semisynthetic derivative of CDCA (0.1–10 μM); GW406, a nonsteroidal FXR ligand (0.01–1 μM); and resiglitazon, a PPARγ ligand (0.1–10 μM) and mRNA expression for FXR, PPARα, α1(1) collagen, SHP, TIMP-1, TIMP-2, MMP-2, and TGFβ1 investigated by quantitative (q)RT-PCR (Fiorucci et al., 2004b).

qRT-PCR. Quantization of the expression level of selected genes was performed by real-time PCR (qRT-PCR) as described previously (Fiorucci et al., 2004b). All PCR primers (Table 1) were designed using the software PRIMER3-OUTPUT using published sequence data obtained from the National Center for Biotechnology Information database. Relative efficiency of the primer used for qRT-PCR was calculated through the determination of standard curves for every gene. Standard curves were performed using standard concentration of cDNA template and estimating the unit of relative fluorescence. Optimization experiments were performed to obtain a primers efficiency value of 100% for every gene.

Western Blot Analysis of FXR and PPARγ Expression on HSCs. Day 0 and day 7 HSCs were incubated with or without 1 μM 6-ECDCA for 24 h at 37°C in Dulbecco’s modified Eagle’s medium. Cell lysates were prepared by solubilization of cells in sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol 2% SDS, and 0.015% bromphenol blue) and separated by polyacrylamide gel electrophoresis. The proteins were then transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) and probed with primary antibodies to FXR and PPARγ (Santa Cruz Biotechnology Inc., Santa Cruz, CA). The anti-immunoglobulin G horseradish peroxidase conjugate (Bio-Rad) was used as the secondary antibody and specific protein bands were visualized using enhanced chemiluminescence (GE Healthcare, Little Chalfont, Buckinghamshire, UK) following the manufacturer’s suggested protocol.

<table>
<thead>
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<th>Gene</th>
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<th>Antisense</th>
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<td>5′-aatacgtgtagaaacccgagga-3′</td>
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</tr>
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<td>5′-gtgtagaagacggaggacgc-3′</td>
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</tr>
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<td>MMP2</td>
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<td>5′-ggtagtacgggtgcgtt-3′</td>
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<td>TIMP1</td>
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<td>Fibronectin1</td>
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<tr>
<td>PPARγ</td>
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<td>α-SMA</td>
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<td>GAPDH</td>
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<td>5′-ggtagtacgggtgcgtt-3′</td>
<td>NM057133</td>
</tr>
</tbody>
</table>

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
In Vivo Studies

All studies were approved by the Animal Study Committee of the University of Perugia. Male Wistar rats (200–250 g) were obtained from Charles River Breeding Laboratories (Portage, MI) and maintained on standard laboratory rat chow on a 12-h light/dark cycle. Three different models of liver fibrosis were used to examine the effect of FXR ligands on PPARγ expression. In the first model, liver fibrosis was induced by repeated intraperitoneal (i.p.) administration of 0.5 ml of porcine serum twice a week for 8 weeks (Fiorucci et al., 2004a). To investigate whether 6-ECDCA was effective in regulating PPARγ expression, porcine serum-administered rats (6–8 each group) were randomized to receive 1 and 3 mg/kg 6-ECDCA via gavage 5 times a week. Control rats were administered 3% carboxymethyl cellulose (CMC) by gavage. At the end of the study, rats were sacrificed under anesthesia with sodium pentobarbital (50 mg/kg i.p.) and terminally bled via cardiac puncture; the liver was removed for examination and blood samples were taken. In the second model, hepatic fibrosis was induced by bile duct ligation (BDL) of 8- to 9-week old male Wistar rats as reported previously (Fiorucci et al., 2004a,b). Sham-operated rats (n = 6) received the same laparoscopic procedure, except that the bile duct was manipulated, but not ligated and sectioned. In total, 24 animals were operated. Two weeks after surgery, surviving rats were randomized to receive placebo, i.e., 3% CMC (six rats) or 6-ECDCA, 3 mg/kg (eight rats) by gavage. Animals were then treated for 14 days. In the third model, liver fibrosis was induced in rats by i.p. injection of CCl4, 100 μl/100 g body weight, in an equal volume of paraffin oil two times a week for 4 weeks. Control rats were injected i.p. with 100 μl/100 g body weight of paraffin oil alone. Rats (six per group) were then treated by oral administration of 3 mg/kg 6-ECDCA in CMC five times a week or 3% CMC alone (control) for 8 weeks.

In another set of experiments, we investigated whether 6-ECDCA interacts with rosiglitazone on liver fibrosis induced by porcine serum administration (Table 1). Groups and duration of treatment are described in Table 2. Animals were followed for 8 weeks.

Liver Histology and Hydroxyproline Determination. For histological examination, portions of the right and left liver lobes (10–15 mg each) from each animal were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with Sirius red (Lopez-De Leon and Rojkind, 1985; Fiorucci et al., 2004a,b). Sham-operated rats (n = 6) received the same laparoscopic procedure, except that the bile duct was manipulated, but not ligated and sectioned. In total, 24 animals were operated. Two weeks after surgery, surviving rats were randomized to receive placebo, i.e., 3% CMC (six rats) or 6-ECDCA, 3 mg/kg (eight rats) by gavage. Animals were then treated for 14 days. In the third model, liver fibrosis was induced in rats by i.p. injection of CCl4, 100 μl/100 g body weight, in an equal volume of paraffin oil two times a week for 4 weeks. Control rats were injected i.p. with 100 μl/100 g body weight of paraffin oil alone. Rats (six per group) were then treated by oral administration of 3 mg/kg 6-ECDCA in CMC five times a week or 3% CMC alone (control) for 8 weeks.

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Statistical Analysis. Analysis of variance followed by Dunnett or Bonferroni correction for multiple comparison was applied when appropriate. EC50 were calculated using Prism III (GraphPad Software Inc., San Diego, CA).

Results

Functional Cooperation between FXR and PPARγ Ligand in HSCs. As shown in Fig. 3A, exposure to FXR ligands, whereas exposure to 1 μM 6-ECDCA, 20 μM CDCA, and 100 nM GW4064 increased PPARα and γ mRNA by 2- to 3-fold (Fig. 1, A–C; n = 6; P < 0.05). Exposure to FXR ligands increased FXR mRNA expression by 1.5-fold and SHP mRNA by 3-fold (Fig. 1, D and E; n = 6; P < 0.05), whereas decreased α(1) collagen mRNA by 60 to 80% (Fig. 1F; n = 6; P < 0.05). Similarly, FXR activation decreased α-SMA and TIMP-1 mRNA by 60 to 80% (data not shown).

Previous studies have shown that PPARγ expression decreases during HSCs trans-differentiation. To investigate whether FXR ligands could revert this pattern, primary cultures of HSCs were grown in plastic dishes for 7 days with or without 6-ECDCA, and expression of NRs was assessed. As shown in Fig. 2, although acquisition of an activated phenotype associates with a slight increase of FXR expression, mRNA, and protein (Fig. 2, A, C, and E; P < 0.05 versus day 1), HSCs trans-differentiation associated with reduced expression of PPARγ mRNA (Fig. 2, B, D, and F; n = 4; P < 0.001). Culturing the cells with 1 μM 6-ECDCA increased PPARγ, mRNA, and protein by 40-fold (Fig. 2, B, D, and F; n = 4; P < 0.01). Similar to 6-ECDCA, both natural and synthetic FXR ligands, CDCA and GW4064 prevented the down-regulation of PPAR-γ caused by HSCs activation (data not shown).

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TABLE 2

Effect of FXR and PPARγ ligands on liver fibrosis induced by 8-week administration of porcine serum to rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats</th>
<th>Final b.wt.</th>
<th>Liver/b.wt.</th>
<th>ALT</th>
<th>ALP</th>
<th>Bilirubin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>376.0 ± 10.5</td>
<td>2.60 ± 0.5</td>
<td>34.3 ± 4.4</td>
<td>22.5 ± 3.6</td>
<td>0.1 ± 0.01</td>
</tr>
<tr>
<td>PS (8 weeks)</td>
<td>10</td>
<td>354.3 ± 12.3</td>
<td>4.1 ± 0.5*</td>
<td>46.4 ± 5.4</td>
<td>29.0 ± 5.2</td>
<td>0.3 ± 0.01</td>
</tr>
<tr>
<td>PS + 6-ECDCA (1 mg/kg)</td>
<td>10</td>
<td>357.3 ± 9.8</td>
<td>3.8 ± 0.3</td>
<td>47.4 ± 2.4</td>
<td>26.6 ± 2.5</td>
<td>0.3 ± 0.01</td>
</tr>
<tr>
<td>PS + 6-ECDCA (3 mg/kg)</td>
<td>10</td>
<td>352.4 ± 12.3</td>
<td>2.8 ± 0.2</td>
<td>47.3 ± 5.3</td>
<td>21.8 ± 5.2</td>
<td>0.2 ± 0.01</td>
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<tr>
<td>PS + rosiglitazone (1 mg/kg)</td>
<td>10</td>
<td>346.9 ± 11.5</td>
<td>4.0 ± 0.3</td>
<td>47.6 ± 2.4</td>
<td>22.4 ± 3.7</td>
<td>0.3 ± 0.06</td>
</tr>
<tr>
<td>PS + rosiglitazone (3 mg/kg)</td>
<td>10</td>
<td>358.3 ± 11.8</td>
<td>2.81 ± 0.4</td>
<td>47.8 ± 4.5</td>
<td>22.4 ± 3.7</td>
<td>0.3 ± 0.07</td>
</tr>
<tr>
<td>PS + 6-ECDCA (1 mg/kg) + rosiglitazone (1 mg/kg)</td>
<td>10</td>
<td>361.4 ± 8.9</td>
<td>3.1 ± 0.5</td>
<td>47.8 ± 3.1</td>
<td>23.8 ± 2.7</td>
<td>0.1 ± 0.02</td>
</tr>
<tr>
<td>PS + 6-ECDCA (3 mg/kg) + rosiglitazone (3 mg/kg)</td>
<td>10</td>
<td>369.6 ± 10.8</td>
<td>2.7 ± 0.4**</td>
<td>37.5 ± 3.3</td>
<td>22 ± 4.2</td>
<td>0.2 ± 0.01</td>
</tr>
</tbody>
</table>

ALP, alkaline phosphatase; ALT, alanine aminotransferase; PS, porcine serum.

* P < 0.05 versus control rats.

** P < 0.05 versus rats administered porcine serum alone.
TGFβ1. Since the concentration of rosiglitazone required to inhibit α1(I) collagen was higher than the EC50 (Jarvinen, 2004) of this agent for the PPARγ, we wondered whether the effect of rosiglitazone was PPARγ-independent. However, exposure to GW9662, a selective PPARγ antagonist fully reversed the effect of rosiglitazone, but not 6-ECDCA, on α1(I) collagen (Fig. 3B; n = 4; P < 0.05 versus rosiglitazone alone).

To investigate whether ligands of FXR and PPARγ might cooperate in repressing α1(I) collagen gene expression, we exposed HSC-T6 to submaximally effective concentrations of the two ligands. As shown in Fig. 3, C and D, although 0.1 μM 6-ECDCA and 1 μM rosiglitazone individually decreased α1(I) collagen and α-SMA mRNA by 30 to 40%, the combination of the two leads to significant increase in this effect, resulting in 3-fold induction of PPARγ and ~80% reduction of α1(I) collagen and α-SMA mRNA (n = 4; P < 0.05 versus 6-ECDCA or rosiglitazone alone). Similarly to rosiglitazone, coinubation of HSCs with 6-ECDCA in combination with pioglitazone and 15-deoxy-Δ12,14-prostaglandin J2 (PGJ2) (Kliewer et al.,1995) i.e., the putative natural ligand of PPARγ, resulted in a significant additive effect in repressing TGFβ1-regulated genes (Fig. 3D; n = 5; P < 0.05 versus pioglitazone or 15-deoxy-Δ12,14-prostaglandin J2 alone).

**In Vivo Activation of FXR Increases Liver PPARγ mRNA.** We then investigated whether in vivo administration of FXR ligands modulates the expression of PPARγ in HSCs. Three different models of liver fibrosis—porcine serum administration, BDL, and CCL4 intoxication—were used for this experiment. As shown in Fig. 4, development of liver fibrosis associates with a significant reduction in the expression of PPARγ that became almost undetectable in HSCs prepared from rats treated with porcine serum or CCL4 for 8 weeks (n = 4 rat/group; P < 0.05 versus control rats). Similarly, expression of PPARγ was nearly undetectable in HSCs obtained 4 weeks after BDL. In contrast to PPARγ, development of liver cirrhosis had no effect on the expression of FXR and SHP mRNA expression. Administration of rats with the FXR ligand resulted in a robust induction of PPARγ expression in all three models. Thus, although treatment with 3 mg/kg 6-ECDCA increased FXR and SHP mRNA by 1.8- to 4-fold, the FXR ligand increased PPARγ mRNA expression by 30- to 50-fold (Fig. 4; P < 0.01 versus cirrhotic rats).

We next tested whether the effects of the FXR and PPARγ ligands on the deposition of liver collagen were additive. Rats administered porcine serum for 8 weeks developed extensive liver fibrosis as demonstrated by morphometric analysis of Sirius red-stained livers, with minimal inflammation and necrosis (Fig. 5B). Accordingly, although no differences in biochemical parameters were observed compared with controls (Table 1). Histological evaluation of liver specimens obtained from rats administered with porcine serum for 8 weeks showed extensive peribular fibrosis resulting in a 10-fold increase of the surface area of hepatic collagen in comparison with control rats (Figs. 5B and 6A; n = 8–12; P < 0.001). The number of α-SMA-positive HSCs in the fibrous septa increased significantly in cirrhotic rats compared with control rats (data not shown; P < 0.001 versus controls).

Quantitative RT-PCR analysis in whole liver homogenates showed a 4- to 10-fold increase in α-SMA, α1(I) collagen, fibronecet, TGFβ1, TIMP-1, and TIMP-2 mRNAs (Fig. 6, C–H; P < 0.01 versus control rats), whereas MMP-2 mRNA was unchanged. Administration of 1 and 3 mg/kg 6-ECDCA and rosiglitazone, respectively, did not affect liver function as measured by plasma alanine aminotransferase, alkaline phosphatase, and bilirubin (P > 0.05 versus control and porcine serum-treated rats). However, both drugs effectively protected rats against development of liver fibrosis at the dose of 3 mg/kg (Figs. 6 and 7). Although expression of selected profibrogenetic markers was reduced in animals...
treated with 1 mg/kg 6-ECDCA (α-SMA, fibronectin, and TGFβ1), this dose slightly reduced the histological score and the liver hydroxyproline content in comparison with animals treated with porcine serum alone. No significant effects were observed in any biochemical or molecular marker of liver fibrosis in animals treated with 1 mg/kg rosiglitazone. In contrast, administration of rats with 3 mg/kg 6-ECDCA or rosiglitazone decreased the area of liver parenchyma occupied by fibrotic tissue and hepatic levels of hydroxyproline as well as expression of α-SMA, α1(I) collagen, fibronectin, TGFβ1, TIMP-1, and TIMP-2 mRNAs by 50 to 60% in comparison with rats administered porcine serum alone (n = 8–12; P < 0.01 versus porcine serum alone; Figs. 6 and 7).

Coadministration of 1 mg/kg 6-ECDCA together with 1 mg/kg rosiglitazone resulted in higher antifibrotic activity with respect to that observed with either of the two drugs alone and reduced the extent of liver fibrosis as measured by morphometric analysis by 50 to 60% in comparison with porcine serum alone (Figs. 5–7; n = 8–12; P < 0.01 versus porcine serum alone).

Similarly, coadministration of 6-ECDCA and rosiglitazone at the dose 3 mg/kg each resulted in a significant potentiation of the antifibrotic effect exerted separately by the two drugs. Indeed, this combination ameliorated the histological score, reduced the liver hydroxyproline content and decreased the expression of α-SMA, α1(I) collagen, fibronectin, TGFβ1, TIMP-1 and TIMP-2 mRNAs by ≈90% (P < 0.05 versus 6-ECDCA and rosiglitazone). The beneficial effect observed in rats treated with the combination of FXR and PPARγ ligands correlated with a significant induction of PPARγ mRNA expression in the liver. Thus, whereas administering rats with 1 mg/kg 6-ECDCA or 1 and 3 mg/kg roglitazione alone increased PPARγ mRNA by 1- to 2-fold, administration of 3 mg/kg 6-ECDCA resulted in 3- to 4-fold induction. Fur-
thermore, the FXR ligand, but not the PPARγ ligand, increased the expression of SHP (3–4-fold) and reduced Cyp7A1 by 70 to 80% (Fig. 7D). Furthermore, similarly to rosiglitazone 6-ECDCA (3 mg/kg) increased the hepatic expression of UCP-2, a PPARγ regulated gene, by 2- to 4-fold ($P < 0.05$ versus control and porcine serum alone).

**Discussion**

FXR and PPARs are ligand-regulated transcription factors that exert multiple regulatory functions on bile acids, glucose, and lipid homeostasis. In the present study, we show that FXR and the three members of the PPAR family are expressed in HSCs and that FXR ligands modulate PPARγ expression, suggesting that an FXR-PPARγ cascade exerts counter-regulatory effects in HSCs. The demonstration that FXR ligands increase PPARα and γ expression is consistent with the finding that PPARα and γ, similar to FXR, function as a braking signal for activation of HSCs and indicates that regulated expression of these NRs has the potential to prevent HSCs transdifferentiation (Friedman, 2003). Indeed, the decrease in PPARγ expression observed at the early stages of HSCs activation and the fact that PPARγ ligands inhibit at least some of the characteristics associated with the activated phenotype of HSCs suggest that PPARγ may be involved in the maintenance of a quiescent phenotype (Galli et al., 2000, 2002; Marra et al., 2000; Kon et al., 2002). In contrast to the effect on PPARα and γ, FXR ligands failed to modulate PPARβ expression. The increased expression of PPARβ, together with the reduced expression of PPARγ observed upon HSCs activation, suggests that a balance between these transcription factors might be responsible for some of the key phenotypic changes responsible for the profibrogenic role of HSCs (Hellemans et al., 2003). In this regard, PPARβ has been shown to act as a potent inhibitor of PPARγ active transcription by the recruitment of corepressors and histone deacetylases to PPARγ-responsive elements (Shi et al., 2002). Thus, the opposite effects that FXR exerts on PPARβ and PPARα and γ suggest that inhibition of HSCs activity caused by its ligands is due, at least in part, to modulation of the ratio of PPARβ versus PPARα or γ.

Previous studies have shown that bile acids regulate PPARα expression in hepatoma cell lines and primary hepatocytes and that a functional FXR response elements is expressed within the human PPARα promoter, which confers responsiveness to FXR ligands (Pineda Torra et al., 2003). Consistent with this finding, we have now shown that 6-ECDCA, a potent FXR ligand, increases rat PPARα mRNA expression in HSCs. Although the exact role of PPARα in regulating HSCs is still undefined, administration of the PPARα agonist Wy-14,643 to mice ameliorates established...
fibrosis and reduces hepatic levels of $\alpha_1(I)$ collagen, TIMP-1, TIMP-2, and MMP-13 (Ip et al., 2004), suggesting that similarly to PPARγ, PPARα might function as a negative regulator of trans-differentiation in HSCs.

Despite the fact FXR and PPARγ are expressed by HSCs and ligands for these receptors decrease the expression of myofibroblastic markers in HSCs (Galli et al., 2000; Marra et al., 2000; Hazra et al., 2004), their expression shows opposite regulation during the process of trans-differentiation. In fact, although the levels of PPARγ mRNA decrease to an almost undetectable level during the process of activation in vitro and in vivo, the expression of FXR remains unchanged (Fig. 2). This differential regulation suggests that although an early reduction in PPARγ plays a permissive role, allowing HSCs to trans-differentiate, the same does not apply to FXR, which regulates the profibrogenetic phenotype at a later stages of activation. Thus, although molecular cross-talk exists between the FXR and PPARγ signaling pathways with FXR ligands inducing PPARγ expression, they seem to affect different regulatory mechanisms.

The ability of FXR ligands to increase PPARγ expression in HSCs raises the question of whether the antifibrotic activity of FXR ligands is mediated through the activation of this nuclear receptor. Experiments carried out with a selective PPARγ antagonist suggest that although a certain overlap between the two signaling pathways exists, inhibition of the profibrogenic activity of HSCs by FXR ligands seems to be largely independent of the effect on PPARγ. Support for this concept comes from the demonstration that although treatment of HSCs with GW9662, a potent and selective PPARγ antagonist (Leesnitzer et al., 2002), fully reversed the inhibition of $\alpha_1(I)$ collagen mRNA caused by rosiglitazone, it was only marginally effective in blocking the inhibitory effect of 6-ECDCA. Together with the fact that 6-ECDCA is 40-fold more potent than rosiglitazone in blocking the up-regulation of $\alpha_1(I)$ collagen induced by TGFβ1, these data indicate that FXR ligands

Fig. 4. In vivo administration of the FXR ligand 6-ECDCA reverts PPARγ mRNA down-regulation in rodent models of liver fibrosis. Fibrosis was induced by 8-week porcine serum administration, BDL (4 weeks), and CCL4 administration for 8 weeks as described under Materials and Methods. 6-ECDCA was administered five times a week for 8 weeks in the porcine serum and CCL4 models and for 2 weeks in the BDL model. HSCs were prepared at the end of these periods of administration as described under Materials and Methods. Data are the mean of at least four animals per group. *, $P < 0.05$ versus control rats. **, $P < 0.05$ versus rats treated with porcine serum, BDL, or CCL4 alone. The effect of 6-ECDCA on FXR, PPARγ, and SHP is shown.
modulate HSCs activity by both PPARγ-dependent and -independent mechanisms.

One important finding of the present study was the demonstration that in vivo treatment with a FXR ligand reverses the down-regulation of PPARγ in HSCs obtained from rats administered porcine serum and CCL4 as well as in BDL rats. Administering rats with 6-ECDCA caused a 2-fold increase in FXR and SHP mRNA, indicating that FXR ligands have the potential to prevent PPARγ down-regulation in these models.

Because thiazolidinediones, rosiglitazone, and pioglitazone effectively prevent HSCs trans-differentiation in vitro and reduce fibrogenesis in rodent model of liver cirrhosis (Galli et al., 2002), it has been suggested that these glucose-lowering agents might be beneficial in treating fibrosis in human disease (Tsukamoto, 2002). Indeed, although clinical studies in human fibrotic diseases are lacking, thiazolidinediones have been used to treat insulin resistance in patients with nonalcoholic steatohepatitis, resulting in a significant improvement of fibrosis as judged by liver histology (Neuschwander-Tetri et al., 2003). However, in contrast to animal models of liver fibrosis, where PPARγ expression is abrogated, it is noteworthy that PPARγ is expressed at elevated levels in the liver of a number of murine models of diabetes or obesity (Bedoucha et al., 2001) suggesting that these conditions (i.e., liver fibrosis and liver steatosis) involve different pathogenetic mechanisms.

Since both our in vivo and in vitro data indicate that FXR ligands can reverse down-regulation of PPARγ, a study was designed to investigate whether a combination of submaximally effective doses of FXR and PPARγ ligands exert an additive effect in protecting against liver fibrosis induced by porcine serum administration (Fiorucci et al., 2004b). Using this model, we demonstrated that although treatment of rats with 1 mg/kg 6-ECDCA and rosiglitazone, i.e., submaximally effective doses of these agents, caused a 20 to 30% reduction of markers of hepatic fibrosis, coadministration of the two ligands reduces liver fibrosis as assessed by liver morphometry by ~60%. Furthermore, although a significant reduction in liver fibrosis was observed in rats treated with 3 mg/kg of both agents, administration of the combination of 6-ECDCA and rosiglitazone at 3 mg/kg for 8 weeks resulted in a 90% reduction in liver collagen content. The reduction in collagen deposition ob-
Obtained by the combination of FXR and PPARγ ligands was associated with a reduction in the parenchymal area occupied by α-SMA-positive cells, suggesting a causal relationship between the decreased number of activated HSCs and the reduced accumulation of extracellular matrix components. Protection against development of liver fibrosis induced by 6-ECDCA associated with a significant induction of PPARγ and SHP gene expression.

Although we have shown that the effect of FXR ligands might be additive to the effects of PPARγ ligands in reducing liver fibrosis, these results extend to other diseases. Indeed, FXR ligands increase the expression of UCP-2, a PPARγ regulated gene involved in regulation of energy metabolism (Matsusue et al., 2003), and suggest that FXR ligands might enhance the glucose-lowering effects of PPARγ ligands.

The synergistic activity of FXR ligand and PPARγ ligand could also contribute to limit the incidence of side effects associated with the use of PPARγ agonist. In fact 6 to 15% of diabetic patient taking rosiglitazone or pioglitazone develop a diuretic-resistant edema (Nesto et al., 2003; Jarvinen, 2004). Since the incidence of side effects caused by these two drugs is dose-dependent, it seems likely that a combination of FXR and PPARγ ligand could contribute to limit the dose of.

Fig. 6. A–F, liver expression of α1(I) collagen, α-SMA, fibronectin, TGFβ1, TIMP-1, and TIMP-2 mRNA in rats administered porcine serum alone or in combination with 6-ECDCA and rosiglitazone (RGT) for 8 weeks. Data are the mean ± S.E. of four to six rats per group. * P < 0.01 compared with control rats. ** P < 0.01 compared with porcine serum alone.
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PPARγ ligand reducing the burden of side effects associated with their use.

In conclusion, the present study demonstrates that FXR ligands regulate PPARγ gene expression and that FXR and PPARγ ligands synergize in regulating profibrogenic activities of HSCs.

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References


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