Characterization of the Species-Specificity of Peroxisome Proliferators in Rat and Human Hepatocytes

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Peroxisome proliferation is a well-defined pleiotropic effect that is mediated by the ligand inducible transcription factor peroxisome proliferator-activated receptor (PPAR) α. Because marked peroxisome proliferation occurs in rodents but not in humans, we aimed to elucidate the molecular and cellular determinants of this species-specificity in hepatocytes. Analysis of peroxisomal marker enzyme activities confirmed that peroxisome proliferators induced acyl-CoA oxidase (ACOX) and to a lesser extent catalase in rat hepatocytes, but not in human hepatoma HepG2 cells. Transient transfection assays revealed that ciprofibrate and Wy 14,643 induced rat but not human PPARα-mediated reporter gene activity in rat FAO and primary hepatocytes on rat but not on human PPARα response elements (PPREs). In contrast, in human HepG2 and primary human hepatocytes, peroxisome proliferators did not induce either human or rat PPARα activity regardless of rat or human PPRE sequences. In addition, no induction of ACOX gene expression was observed in human hepatocytes independent of the expression level of human PPARα. Remarkably, no distinct peroxisome proliferation related responses were observed in human hepatocytes when rat PPARα was transfected, although human hepatocytes were responsive to PPARα-mediated induction of carnitine palmitoyl transferase-1A and 3-hydroxy-3-methylglutaryl-CoA synthase. These results confirmed that PPARα and PPREs are important determinants for the species-specificity of peroxisome proliferation. Nevertheless, our results showed that human hepatocytes limit the extent of peroxisome proliferation regardless of PPARα expression.

Key Words: PPARα; HepG2; FAO; primary hepatocytes; fibrates; Wy 14643.

Peroxisome proliferators are a diverse group of chemicals that cause pleiotropic effects including proliferation of peroxisomes that is accompanied by induction of peroxisomal enzyme expression (Reddy and Chu, 1996). These pleiotropic effects are mediated by activation of the peroxisome proliferator-activated receptor (PPAR) α, as shown by the phenotypes of PPARα knock-out mice (Corton et al., 2000; Lee et al., 1995; Willson, 2000). PPARα functions as a ligand-inducible transcription factor for genes involved in mitochondrial and peroxisomal metabolism (Corton et al., 2000) and it is well established that marked species differences in response to peroxisome proliferators exist: Rodents show high peroxisomal enzyme induction while humans do not (Vanden Heuvel, 1999).

These species differences in PPARα function have been the focus of several research papers (reviewed in Cattley et al., 1998). The functions of rat and human PPARα are similar and homology of the DNA binding domain and ligand binding domain is high (Goettlicher et al., 1992; Mukherjee et al., 1994; Sher et al., 1993); however, human hepatocytes display only weak induction of marker enzyme activity like acyl CoA oxidase (ACOX). Several determining factors for the different activities of PPARα in rat vs. human hepatocytes have been identified. One factor might be due to the lower expression levels of PPARα in human liver compared to rats (Tugwood et al., 1996) and a second factor might be the existence of an inactive PPARα splice variant in human liver samples (Palmer et al., 1998). Another factor has been shown to be the responsiveness of PPARα regulated genes that is defined by PPAR response elements (PPRE) located within the promoter region of target genes. Both human and rat ACOX gene promoter contain the consensus PPRE half site TGACCT and a second nonconsensus half-site (Tugwood et al., 1992; Varanasi et al., 1996, 1998). Comparison of the rat and human promoter sequences revealed that the human ACOX PPRE did not mediate PPARα activity; however, human PPARα displayed activity on the rat ACOX promoter (Hasmall et al., 2000; Lambe et al., 1999) and was able to induce endogenous genes involved in lipid metabolism (Lawrence et al., 2001).

Our aim was to determine whether PPARα and the PPRE sequences are sufficient to account for these species differences. We, therefore, analyzed the contribution of PPARα, PPRE and in particular how and to what extent the cellular environment may determine PPARα-mediated responses in rat and human hepatocytes. First, marker enzyme inductions in rat primary hepatocytes, the rat hepatoma FAO, and the human hepatoma HepG2 cell line were investigated. In transient trans-
fection studies we then examined whether human or rat PPARα exert activity on a rat or human PPRE derived from the ACOX promoter in rat and human hepatocytes. Subsequently, we measured expression of endogenous peroxisomal marker genes as well as of human PPARα responsive genes in rat and human primary hepatocytes in the presence or absence of transfected rat or human PPARα. This comprehensive analysis revealed that all parameters investigated determined the species-specificity of peroxisome proliferation and that human hepatocytes limited PPARα activity.

MATERIALS AND METHODS

Materials and chemicals. Media, serum, supplements, enzymes, and biochemicals were purchased from Sigma (Deisenhofen, Germany) unless otherwise stated. HepG2 and FAO cells were obtained from ECACC (Salisbury, UK). Steroid deprived dextran-coated charcoal stripped fetal bovine serum (DCC/FBS) was from HyClone (Lot AKD11642A, Perbio Science, Bonn, Germany). Primary male hepatocytes were obtained from In Vitro Technologies (Order number M00005, Lot MSE, Baltimore, MD). Ciprofibrate, clotibofibrate, and pirinixic acid (Wy 14,643) were from Sigma-Aldrich (Deisenhofen, Germany).

Cell culture. HepG2 and FAO cells were cultured in Dulbecco’s modified Eagle medium (DMEM)/F12 supplemented with 10% FBS and 5% FBS, respectively, 1 mM sodium pyruvate and antibiotics. Cells were cultured at 37°C/5% CO2 in air in a humidified atmosphere.

Isolation of primary hepatocytes from rat liver. Primary rat hepatocytes were prepared freshly from male Wistar rats (200–250 g) by the in situ perfusion procedure (Seglen, 1973, 1976). Livers were perfused with 0.5 mg/ml collagenase, the liver capsule removed, and the released hepatocytes filtered through a 250 μm mesh followed by a second filtration step using a 100 μm mesh nitex membrane. The filtered hepatocytes were resuspended in phenol-red free DMEM/F12 supplemented with 10% DCC/FBS and 5 μg/ml insulin. The cell number and viability of the suspension was assessed by trypsin blue exclusion. Fifty μl trypsin blue was mixed with 50 μl cell suspension and the number of viable cells (cells that were not stained with trypsin blue) were determined in a hemacytometer. Primary cell cultures that had a viability of more than 80% were used for the experiments. For better attachment and viability of primary hepatocytes, tissue culture plates were coated with 6 μg/ml collagen I. For attachment, rat hepatocytes were cultured at 37°C/5% CO2 in air in a humidified atmosphere for at least 3 h prior to the experiments.

Thawing of cryopreserved human hepatocytes. Cryopreserved human hepatocytes were thawed according to the procedure provided by the manufacturer (In Vitro Technologies, Baltimore, MD). Briefly, two vials of cryopreserved human hepatocytes were rapidly thawed and poured into 40 ml of phenol-red free DMEM/F12 supplemented with 10% DCC/FBS and centrifuged at 60 × g for 5 min. The cell pellet was resuspended in 5 ml phenol-red free DMEM/F12 supplemented with 10% DCC/FBS and 5 μg/ml insulin. The cell number and viability was assessed as described for rat hepatocytes and primary cell cultures that had a viability of more than 80% were used for the experiments. Primary hepatocytes were seeded on collagen-coated plates. For attachment, human hepatocytes were cultured at 37°C/5% CO2 in air in a humidified atmosphere for at least 3 h prior to the experiments.

Cytotoxicity assay. For assessing cytotoxicity and cell proliferation induced by the test compounds the WST-1 kit was used according to the protocol provided by the manufacturer (Roche, Mannheim, Germany). This test measures the enzymatic formation of formazan. The formazan formation correlates with cellular enzyme activity and cell number. Therefore, a decrease in formazan formation and hence absorption indicates cytotoxicity. FAO and HepG2 cells were seeded at a density of 0.5 × 10⁴ cells/cm² while 2 × 10⁴ primary cells/cm² were seeded in 96-well plates. IC₅₀ values (compound concentration yielding 50% inhibition of formazan formation) were derived by nonlinear curve-fitting of dose-response curves using Origin software (Microcal Software, Northampton, MA) and are given as mean ± SD of at least three independent experiments.

Protein extraction and determination for enzyme activity assays. Fifteen hours prior to treatment, cells were seeded in culture medium. FAO and HepG2 cells were seeded at a density of 0.2 × 10⁵ cells/cm² while 2 × 10⁴ primary cells/cm² were seeded in tissue culture plates. Then, test compounds were added (vehicle was dimethyl sulfoxide [DMSO] at 0.1% volume per volume [v/v] final concentration). The medium was changed every 24 h during treatment. The cells were harvested in ice cold PBS buffer and lysed in 0.1% (v/v) Triton X-100 in PBS buffer. Protein concentrations were determined according to Bradford (1976) with bovine serum albumin as standard.

Acyl-CoA oxidase (ACOX) activity. Palmitoyl-CoA oxidase activity was assayed by determining the production of hydrogen peroxide using a modified photometric detection method (Small et al., 1985). The reaction medium contained 11 mM phosphate buffer and 40 mM aminotriazol buffer at pH 7.4, 0.8 mg horseradish peroxidase E-A, 0.05 mM leuco-dichlorodihydro-fluoresceine diacetate and cell homogenate containing 50 to 200 μg of protein in 300 μl. The reaction was started by the addition of 10 μl of 3 mM palmitoyl-CoA lithium salt solution in 11 mM phosphate buffer pH 7.4. The enzyme kinetic was measured for 10 min at 490 nm.

Catalase activity. The catalase activity was determined by a photometric method (Hübli and Breitschneider, 1964). The assay was performed at 4°C. The reaction mixture (200 μl) contained 20 mM imidazole-HCl buffer pH 7, 0.1% weight per volume (w/v) delipidated bovine serum albumin, and 1.5 mM hydrogen peroxide. The reaction was started by the addition of the cell homogenate (5–40 μg protein/ml) and stopped with 100 μl of 2 M sulfuric acid containing 0.125 % (w/v) titanium (IV) sulfate. The remaining hydrogen peroxide was assayed at 412 nm by the absorption of peroxotitanium sulfate.

Carnitine-acyltransferase activity. Carnitine-acyltransferase (CAT) activity was determined according to (Bieber and Markwell, 1981) with slight modifications. The reaction volume was 200 μl containing 125 mM Tris-HCl buffer pH 8.0, 2.5 mM ethylenediaminetetraacetic acid, 0.5 mM diethionitrobenzenoacid (DTNB), 0.2 mM acetyl-CoA, and 20–50 μg protein. The reaction was started by addition of 5 mM L-carnitine. The DTNB complex was measured for 10 min at 412 nm.

Generation and characteristics of expression and reporter plasmids. The mammalian expression plasmids for human and rat PPARα (pSG5-3pPPARα and pSG5-TPPARα, respectively) were kindly provided by W. Wahl (IBA, Geneva, Switzerland). The mammalian expression plasmid for human RXRα (pcDNA4-HRXRα) was generated using standard cloning procedures (Ausubel et al., 1998). The full-length cDNA of human RXRs (GenBank accession number NM_002957; nucleotide positions 55–1559) was cloned into pcDNA4-HisMAX (Invitrogen, Karlsruhe, Germany) using the TOPO TA cloning kit (Invitrogen, Karlsruhe, Germany) and total RNA isolated from human liver samples. The correct sequence of the cDNA was verified by sequencing. Firefly luciferase reporter plasmids were generated by placing three copies of the rat (Tugwood et al., 1992) or human (Varanasi et al., 1996, 1998) PPARα response element derived from the ACOX promoter (rat ACOX PPRE: TGACCTGTGGTCCT and human ACOX PPRE: AGGTGACTGTGGTCA) in the multiple cloning site of the pGL3-SV40 reporter plasmid (Promega, Mannheim, Germany) and named pGL3-SV40-3xPPRE and pGL3-SV40-3xPPRE, respectively. The luciferase reporter containing the full-length rat ACOX promoter (pGL3-rACOX) was kindly provided by J. Tugwood (AstraZeneca, UK; Tugwood et al., 1992).

Transient transfection and transactivation assay. HepG2 and FAO cells were seeded on 24-well plates 15 h prior to transfection in phenol-red free DMEM/F12 supplemented with 10% and 5% DCC/FBS, respectively. 0.2 × 10⁴ viable primary hepatocytes were dispensed well in collagen-coated 24-well plates in phenol-red free DMEM/F12 supplemented with 10% DCC/FBS and
5 µg/ml insulin as described above. The plasmids were transfected in phenol-red free DMEM/F12 supplemented with 5% DCC/FBS using Fugene 6 (Roche, Mannheim, Germany) according to the protocol provided by the manufacturer. Each well received 0.1 µg empty pSG5, pSG5-hPPARα or pSG5-rPPARα, 0.1 µg of pcDNA4-hRXRα, 0.5 µg of firefly luciferase reporter and 0.01 µg pRL-CMV (Renilla luciferase for normalization; Promega, Mannheim, Germany). After transfection, cells were treated with test compounds (final concentration of vehicle DMSO 0.5% v/v) for 22 h in medium supplemented with 10% DCC/FBS as described above. Luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega, Mannheim, Germany) according to the protocol provided by the manufacturer. Each value was normalized for cell number and transfection efficiency to the luciferase control (pRL-CMV) and each data point obtained represents the average of duplicate determinations. All experiments were repeated at least three times. EC50 values (ligand concentration yielding half-maximal activation) were derived by non-linear curve-fitting of transactivation curves using Origin software (Microcal Software, Northampton, MA) and are given as mean ± SD of at least three independent experiments. Control transfections were performed using 0.5 µg empty pGL3-basic (Promega, Mannheim, Germany) instead of reporter vector per well. Transfection efficiencies were highest in HepG2 cells (average of four tests: 20,000 light units of pRL-CMV per well) and were in FAO approximately 25% compared to HepG2 (average of four tests: 5000 light units of pRL-CMV per well). Primary rat hepatocytes displayed transfection efficiencies of 3000 light units of pRL-CMV per well (average of four tests), and primary human hepatocytes displayed transfection efficiencies of 2000 light units of pRL-CMV per well (average of three tests).

**RNA extraction and isolation.** The RNA was isolated according to the instructions given by the manufacturer (QIAGEN RNaseasy® Mini Kit, Hilden, Germany) using additionally a QIAshredder spin column (QIAGEN, Hilden, Germany) and DNA digestion (QIAGEN DNase Kit, Hilden, Germany).

**Real-time quantitative PCR.** The purified RNA was subjected to reverse transcription using random hexamers and TaqMan® reverse transcription reagents (Applied Biosystems, Weiterstadt, Germany) according to the protocol provided by the manufacturer. The primer and probes used for the real-time PCR assay were designed using the PrimerExpress software (Applied Biosystems, Weiterstadt, Germany) and oligonucleotides were synthesized by Applied Biosystems (Weiterstadt, Germany). Target genes with sequences of primer and probes used are listed in Table 1. The probes were all labeled with the fluorescent dyes FAM (6-carboxy-fluorescein) and TAMRA (6-carboxy-tetramethyl-rhodamin) at the 5‘- and 3‘-end, respectively (Applied Biosystems, Weiterstadt, Germany). Real-time PCR was performed on an Applied Biosystems ABI Prism 7000 Sequence Detection System. The primer concentration was optimized prior to use with a fixed probe concentration of 200 nM in a TaqMan® Universal PCR buffer (Applied Biosystems, Weiterstadt, Germany) in 25 µl reaction volume per well. All samples were run in triplicates and a standard curve from 2.5 to 20 ng cDNA was generated for each experiment. Ten ng of sample cDNA was used and normalized to 18S ribosomal RNA (rRNA) control (for human and rat 18S rRNA pre-developed TaqMan assay reagents [PDAR] were used; Applied Biosystems, Weiterstadt, Germany). Forty-one cycles were run with the following parameters: 2 min at 50°C, 10 min at 95°C and for each cycle 15 s at 95°C for denaturation and 1 min at 60°C for transcription. Two different negative controls were performed, one omitting the reverse transcription step and one omitting target RNA. Assays were evaluated only when the negative controls did not show any amplification products.

**Statistical analysis.** Statistical analyses were performed with the XLStat software (Addinsoft, Paris, France). Significant differences of treated samples compared to vehicle control (Figs. 1–6) were determined using ANOVA followed by Dunnett’s test (n = 3, p < 0.05). Statistical significance (n = 3, p < 0.05) based on pair-wise comparison in Figures 3 and 4 were determined using ANOVA followed by Tukey’s test.

**RESULTS**

**Cytotoxicity.**

We investigated the species specificity of peroxisome proliferators in rat and human hepatocytes as well as in rat hepatoma FAO and human hepatoma HepG2 cells. For the assessment of cytotoxicity, we determined IC50 values in FAO, HepG2, and primary rat and human hepatocytes treated with peroxisome proliferators (Table 2). Wy 14,643 was more cytotoxic than the fibrates and the primary hepatocytes were less sensitive to the cytotoxic effects of peroxisome proliferators than FAO and HepG2 cells (Table 2).

**Peroxisome Proliferators Induced Marker Enzyme Activity in Rat Hepatocytes But Not Human HepG2 Cells**

A hallmark physiological response in rodent peroxisome proliferation is the induction of the peroxisomal enzymes ACOX and carnitine-acyltransferase (CAT). Catalase, the enzyme that detoxifies peroxide produced by ACOX is usually induced to a lower extent in rodents and rat hepatocytes (Reddy and Chu, 1996). We therefore analyzed whether clobibrate and ciprofibrate induced a distinct enzyme induction pattern in primary rat hepatocytes, rat FAO, or human HepG2 cells (Fig. 1; Duclos et al., 1997; Perrone et al., 1998; Stangl et al., 1995). We first performed dose-response and time-course experiments with clobibrate and ciprofibrate (data not shown). Maximum inductions were observed with 250 µM fibrates treated for 72 h and are shown in Figure 1. As expected, fibrates strongly induced ACOX and CAT in primary rat hepatocytes whereas FAO cells showed lower but distinct induction of ACOX and CAT (Figs. 1A and 1B). In contrast, catalase was weakly induced (maximum of 2.2-fold induction in rat hepatocytes when treated with ciprofibrate; see Fig. 1C). The human HepG2 cells exerted only slight inductions of ACOX and CAT activity that reached significance for ciprofibrate treatment (Fig. 1).

**PPARα, PPRE, and the Cellular Environment Determine the Different Transactivation in Rat Compared to Human Hepatocytes**

We further examined whether the species-specific activity of peroxisome proliferators were due to rat or human PPARα or the promoter sequences. For this purpose, FAO, HepG2, as well as rat and human primary hepatocytes were transfected with either rat or human PPARα expression vectors. As reporter genes we used either a rat (Tugwood et al., 1992) or human (Varanasi et al., 1996, 1998) ACOX PPRE or the rat full-length ACOX promoter (Tugwood et al., 1992). Control transfections were performed with a reporter vector lacking PPRE sequences that did not show any induced activity (data not shown). Wy 14,643 as well as ciprofibrate induced a dose-dependent increase of full-length rat ACOX and rat ACOX PPRE reporter gene activity in rat primary hepatocytes.
and rat FAO cells, but not in primary human hepatocytes or HepG2 cells (data not shown). The potencies (EC_{50}) of Wy 14,643 and ciprofibrate to induce PPAR{\alpha} were comparable in rat hepatocytes and FAO on both rat ACOX PPRE (3'-PPRE) and the full-length ACOX promoter (Table 3).

We then compared the basal normalized activities of transfected rat or human PPAR{\alpha} on the rat ACOX PPRE in the different cell lines (Fig. 2). Transfection of rat PPAR{\alpha} yielded a distinct—although statistically not significant—4.2-fold induction of the rat ACOX PPRE in FAO and a strong and significant 20-fold induction in rat primary hepatocytes in the absence of ligand compared to transfection of empty vector (Fig. 2). No significant effects of either rat or human PPAR{\alpha} were observed in human HepG2 and human primary hepatocytes (Fig. 2). Comparable results were obtained with the full-length rat ACOX promoter, whereas the human ACOX PPRE showed no increased normalized activity when either rat or human PPAR{\alpha} were transfected in all cell lines tested (data not shown).

For comparison of the induction of rat vs. human ACOX

<table>
<thead>
<tr>
<th>Target (Genbank accession no.)</th>
<th>Sequence (shown from 5' to 3' end)</th>
<th>Position (nucleotides)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human ACOX (NM_004035)</td>
<td>For. primer: GCCTATGCTTCCAGTTTG</td>
<td>1065–1086</td>
</tr>
<tr>
<td></td>
<td>Rev. primer: TTGCAGTCCAGGAGTGAAAG</td>
<td>1209–1230</td>
</tr>
<tr>
<td></td>
<td>Probe: ACATGAAGAGACCTATACCCGGAT</td>
<td>1088–1118</td>
</tr>
<tr>
<td>Human catalase (XM_030346)</td>
<td>For. primer: AAGATGGCATTACACCCTTTG</td>
<td>1584–1607</td>
</tr>
<tr>
<td></td>
<td>Rev. primer: TTACCGGATGAAAGCTAAGCTT</td>
<td>1701–1724</td>
</tr>
<tr>
<td></td>
<td>Probe: TCCGGATCTACTCTGGGGGCA</td>
<td>1611–1632</td>
</tr>
<tr>
<td>Human PCNA (NM_002592)</td>
<td>For. primer: TCCGCCACATGTTCA</td>
<td>110–127</td>
</tr>
<tr>
<td></td>
<td>Rev. primer: TATCCAGACGGCTCTGTT</td>
<td>206–225</td>
</tr>
<tr>
<td></td>
<td>Probe: CGCCTGTCAGGGCTCCATC</td>
<td>131–152</td>
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<tr>
<td>Human PPAR\alpha (NM_005035)</td>
<td>For. primer: CGTGCTTCTCCTGTTTACTAGTA</td>
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</tr>
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<td></td>
<td>Rev. primer: CACATCGGACACAGATG</td>
<td>218–236</td>
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<td></td>
<td>Probe: TGGAGCTCGGCCACACAACA</td>
<td>176–197</td>
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<tr>
<td>Human CPT1A (NM_001876.1)</td>
<td>For. primer: TGTTTTACAGGGCCAAACTG</td>
<td>915–935</td>
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<td></td>
<td>Rev. primer: TGGAAATCGTGATCCCCAAA</td>
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<td></td>
<td>Probe: ACCGGGAGAAATACAAACATTGTC</td>
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<td>Human apo CIII (M28614.1)</td>
<td>For. primer: CAGCTCTATGGCGGCTACA</td>
<td>117–137</td>
</tr>
<tr>
<td></td>
<td>Rev. primer: ACGTGTCGTCAGTGCATCTT</td>
<td>179–198</td>
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<td></td>
<td>Probe: AAGCACAGCAGAAGACCGCC</td>
<td>139–160</td>
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<td>Human HMGCS (X83618.1)</td>
<td>For. primer: AGCAAGTTTCTTTTCTTTCAGAT</td>
<td>1302–1329</td>
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<td></td>
<td>Rev. primer: GATGTTCTGGACACAACTTG</td>
<td>1379–1401</td>
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<td></td>
<td>Probe: CAGGATGTCTCGGCTAGTTCCC</td>
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<td>Rat ACOX (NM_017340)</td>
<td>For. primer: GTGATTAGCGCAGACATTGGA</td>
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<td>Rev. primer: TCGTCAGAATGTCCTCAATTTTC</td>
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<td></td>
<td>Probe: AGTCCGAGAGAATCCCGGCCG</td>
<td>149–171</td>
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<td>Rat catalase (NM_012520)</td>
<td>For. primer: TTATGCGCTCCAGATTTTTC</td>
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<tr>
<td></td>
<td>Rev. primer: ACCTTGTGCAAGTTCAGT</td>
<td>988–1010</td>
</tr>
<tr>
<td></td>
<td>Probe: ATGCCATGGGCAAGTTACACC</td>
<td>883–907</td>
</tr>
<tr>
<td>Rat PCNA (NM_022381)</td>
<td>For. primer: ACGTCTCTTATTGCGACTTACTC</td>
<td>193–218</td>
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<td></td>
<td>Rev. primer: TAATGAGTCTTATTACACACATC</td>
<td>327–353</td>
</tr>
<tr>
<td></td>
<td>Probe: ATCGCAACCTCGGACTG</td>
<td>250–270</td>
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<tr>
<td>Rat PPAR\alpha (NM_013196)</td>
<td>For. primer: TGGAGTCCACCGCATGTAAG</td>
<td>719–739</td>
</tr>
<tr>
<td></td>
<td>Rev. primer: TGTCCCAGGTCTTCTTGAATCT</td>
<td>831–855</td>
</tr>
<tr>
<td></td>
<td>Probe: CTCTTCTCGGCGAAATCTCGGCAATACC</td>
<td>749–778</td>
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<td>Rat CPT1A (NM_031559)</td>
<td>For. primer: GGTTCAAGAAGTGGCATTACACT</td>
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<td></td>
<td>Rev. primer: TCACACCCACACCGAGA</td>
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<td></td>
<td>Probe: TGTTCCCCGGAATCCGT</td>
<td>160–179</td>
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<td>Rat apo CIII (J02596)</td>
<td>For. primer: TGCAAGGGCTACATGGGAACAA</td>
<td>94–114</td>
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<td></td>
<td>Rev. primer: CAGCTATATCGACTCTGCTAGCT</td>
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<td></td>
<td>Probe: CCTCAAGACGGCTGGAGTCCAAAG</td>
<td>116–143</td>
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<tr>
<td>Rat HMGCS (M33648.1)</td>
<td>For. primer: TGCGTGTCATCTTCCA</td>
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<td></td>
<td>Rev. primer: TGCGTGTCATCTTCCA</td>
<td>1550–1568</td>
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<tr>
<td></td>
<td>Probe: AACCTCTTCCAGGGACTTGGTACCTTGA</td>
<td>1499–1528</td>
</tr>
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</table>
Peroxisome proliferators in hepatocytes

**TABLE 2**

Cytotoxicity of Peroxisome Proliferators

<table>
<thead>
<tr>
<th>Primary hepatocytes</th>
<th>IC₅₀ (µM)ᵃ</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Rat</td>
</tr>
<tr>
<td>Wy 14,643</td>
<td>248 ± 18</td>
</tr>
<tr>
<td>Clofibrate</td>
<td>1457 ± 85</td>
</tr>
<tr>
<td>Ciprofibrate</td>
<td>891 ± 96</td>
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</tbody>
</table>

*IC₅₀ values (concentration resulting in 50% inhibition of formazan formation) were derived by nonlinear curve-fitting of dose-response curves using Origin Software (Microcal Software, Northhampton, MA) and are given as mean ± standard deviation of at least three independent experiments.

**TABLE 3**

Potency of Peroxisome Proliferators to Induce Rat PPARα Mediated Activity

<table>
<thead>
<tr>
<th>Rat primary hepatocytesᵇ</th>
<th>Full-length ACOX</th>
<th>ACOX PPRE</th>
<th>FAOᶜ</th>
<th>Full-length ACOX</th>
<th>ACOX PPRE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wy 14,643</td>
<td>26 ± 3.1</td>
<td>28.2 ± 4.7</td>
<td>31 ± 2.2</td>
<td>33 ± 2.8</td>
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</tr>
<tr>
<td>Ciprofibrate</td>
<td>35 ± 6.5</td>
<td>26.4 ± 2.2</td>
<td>32 ± 4.6</td>
<td>27 ± 0.6</td>
<td></td>
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</tbody>
</table>

*EC₅₀ values (ligand concentration yielding half-maximal activation) were derived by nonlinear curve-fitting of transactivation curves using Origin Software (Microcal Software, Northhampton, MA) and are given as mean ± standard deviation of three independent experiments.

**RESULTS**

PPARα by either rat or human PPARα in the presence of ligand, the maximum normalized fold inductions compared to the vehicle control in FAO (Fig. 3A) and HepG2 (Fig. 3B) are shown. The rat PPARα induced only the rat PPRE or the rat ACOX promoter in FAO cells, whereas the human PPARα did not produce any significant transactivation on the human PPRE in HepG2 cells when treated with peroxisome proliferators (Fig. 3). The human PPARα was only slightly active on either human (1.6 ± 0.4 fold induction; Fig. 3A) or rat PPRE (1.8 ±

**FIG. 1.** Induction of ACOX, CAT, and catalase activity in primary rat hepatocytes, FAO, and HepG2 cells by clofibrate and ciprofibrate. Primary rat hepatocytes, FAO, and HepG2 cells were treated for 72 h with 250 µM clofibrate (solid columns) or ciprofibrate (striped columns). (A) ACOX, (B) CAT, and (C) catalase activity was measured as described in the Materials and Methods section. Open columns indicate the values for the vehicle control. Shown are mean values with standard deviation of three independent experiments. Fold (x) inductions compared to vehicle control are indicated on top of each column. Significant differences of treated samples compared to vehicle control were determined using ANOVA/Dunnett’s test (n = 3, p < 0.05) and are indicated by an asterisk.

**FIG. 2.** Normalized activities on the rat ACOX PPRE reporter gene in the presence of transfected rat or human PPARα in FAO, HepG2 cells, and primary rat and human hepatocytes. FAO, HepG2 cells, and primary rat and human hepatocytes were transiently transfected with mammalian expression vectors for human RXRα (pDNA4-hRXRα), normalization vector pRL-CMV and, as indicated, with empty vector (pSG5; striped columns) or mammalian expression plasmids for either human (pSG5-hPPARα; open columns) or rat PPARα (pSG5-rPPARα; solid columns) along with rat ACOX PPRE-reporter (pPRE). Following transfection, cells were treated with vehicle (0.5 % DMSO) for 24 h. Each value was normalized for cell number and transfection efficiency to the internal luciferase pRL-CMV control. Each data point represents the mean value with standard deviation of at least three independent experiments. Fold (x) inductions compared to pSG5-transfected cells (striped columns) are indicated on top of each column. Significant differences of cells transfected with either rat or human PPARα compared to cells transfected with empty pSG5 vector were determined using ANOVA/Dunnett’s test (n = 3, p < 0.05) and are indicated by an asterisk.
compared to endogenous PPARα (Fig. 3A). No distinct PPARα-mediated activity was observed in HepG2 cells, regardless of PPARα or PPRE used (Fig. 3B).

Since the effects observed in FAO and HepG2 cells could be due to the transformed cell type and may not reflect the physiology in hepatocytes, we performed the same experiments in primary rat and human hepatocytes (Fig. 4). The rat primary hepatocytes facilitated marked peroxisome proliferator induced activity of rat PPARα on rat ACOX PPRE or the full-length ACOX promoter only (Fig. 4A). In contrast to the results obtained in FAO cells (Fig. 3A), induction of the full-length ACOX promoter was not increased by transfection of rat or human PPARα into rat primary hepatocytes (Fig. 4A), which is likely due to high level of endogenous PPARα expression (see below) and/or the high basal activity of transfected rat PPARα in primary rat hepatocytes (Fig. 2). In agreement with the results obtained in HepG2 cells (Fig. 3B), we did not observe any distinct PPARα-mediated activity in human hepatocytes (Fig. 4B). Surprisingly, transfected rat PPARα did not increase the induction of rat ACOX PPRE or ACOX promoter by Wy 14,643 or ciprofibrate in human hepatocytes (Fig. 4B), although the transfection efficiencies of rat and human PPARα were comparable for primary rat and human hepatocytes (see Materials and Methods section).

**PPARα mRNA Is Highly Abundant in Rat But Not in Human Hepatocytes**

Since rat but not human hepatocytes showed PPARα-mediated activity (Figs. 2 and 4), we analyzed the basal mRNA expression.
expression levels of endogenous PPARα in the rat and human cell lines used (Table 4). As expected, the basal expression level of endogenous PPARα in rat FAO cells was more than 4-fold higher compared to human HepG2 cells. Rat primary hepatocytes showed the highest basal expression levels of PPARα whereas HepG2 and human primary hepatocytes had similar, low basal expression levels of human PPARα (Table 4).

**Human Hepatocytes Remain Refractory to Peroxisome Proliferator Induced Gene Expression Independent of Rat or Human PPARα Expression Level**

In order to investigate PPARα-mediated endogenous gene expression by peroxisome proliferators, we quantified mRNA expression of ACOX, catalase, and the proliferation marker PCNA by TaqMan® analysis. The promoter region of the genes coding for ACOX and catalase contain PPREs (Girnun et al., 2002; Tugwood et al., 1992; Varanasi et al., 1996), but it is not known whether the PCNA promoter contains a PPRE. We first analyzed the effects of endogenous PPARα in the cell lines investigated when treated with noncytotoxic doses of Wy 14,643 and ciprofibrate that yielded maximum inductions in transactivation assays (Figs. 3 and 4). Gene expression was analyzed at 2, 24, and 72 h and normalized to 18S rRNA. Consistent with the enzyme induction data (Fig. 1), no significant catalase induction was observed in HepG2 or human hepatocytes (data not shown). In primary rat hepatocytes and FAO cells, catalase was induced about 2-fold and the proliferation marker PCNA was not induced at all in either rat or human cells tested (data not shown). In contrast, the well-defined marker gene for peroxisome proliferation ACOX was highly induced in FAO and rat hepatocytes after 24 and 72 h, respectively, upon treatment with Wy 14,643 or ciprofibrate (Fig. 5) consistent with the enzyme induction data (Fig. 1) and the transactivation experiments (Figs. 3 and 4).

ACOX expression was weakly, but significantly induced in HepG2 cells treated with Wy 14,643 and primary human hepatocytes treated with Wy 14,643 and ciprofibrate for 24 h compared to the vehicle control (Fig. 6). All other treatments did not induce any significantly increased expression of ACOX in HepG2 cells or in human hepatocytes compared to the vehicle control (Fig. 6).

It is well established that rat hepatocytes respond to peroxisome proliferators by induction of ACOX whereas human hepatocytes remain refractory (Lawrence et al., 2001). We have therefore extended our study and investigated the effects of both endogenous PPARα and transfected rat or human PPARα on the expression of ACOX and other rat and human PPARα responsive genes, i.e., 3-hydroxy-3-methylglutaryl-CoA synthase (HMGCS), carnitine palmitoyl transferase-1A (CPT1A), and apolipoprotein CIII (apo CIII; Brandt et al., 1998; Hsu et al., 2001; Lawrence et al., 2001; Rodriguez et al., 1994; Staels et al., 1995). Based on the time-course experiments of ACOX expression (Figs. 5 and 6), we treated FAO, HepG2, primary rat and human hepatocytes for 24 h with 100 μM Wy 14,643 and analyzed PPARα target gene expression (Figs. 7–9). Transfection of rat and human hepatocytes as well as hepatoma cells with human or rat PPARα cDNA yielded a

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**TABLE 4**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Basal PPARα expression (% 18S)</th>
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<tbody>
<tr>
<td>FAO</td>
<td>0.2 ± 0.02</td>
</tr>
<tr>
<td>HepG2</td>
<td>0.05 ± 0.02</td>
</tr>
<tr>
<td>Primary rat hepatocytes</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>Primary human hepatocytes</td>
<td>0.08 ± 0.03</td>
</tr>
</tbody>
</table>

*Note. Basal mRNA expression was quantified by real-time quantitative PCR using TaqMan® analysis as described in the Materials and Methods section.*

*mRNA expression is expressed as % of 18S rRNA quantity and calculated from three different RNA preparations. Values are given as mean with standard deviation.*
distinct increase of human or rat PPARα mRNA expression, respectively (Figs. 7 and 8). Furthermore, transfected rat PPARα increased basal ACOX expression levels in FAO (Fig. 7A) but not in primary rat hepatocytes (Fig. 8A), which is likely due to the higher endogenous PPARα expression in primary rat hepatocytes compared to FAO cells (Table 4). Wy 14,643 distinctively induced ACOX gene expression in mock and rat PPARα transfected FAO as well as in rat hepatocytes (Figs. 7A and 8A). Increased human PPARα mRNA expression—up to similar levels as observed for rat PPARα in rat hepatocytes (Figs. 7 and 8)—as well as overexpression of rat PPARα mRNA did not distinctively increase the susceptibility to ACOX induction by Wy 14,643 in HepG2 or human hepatocytes (Figs. 7B and 8B).

We then analyzed the expression of the well-characterized human PPARα responsive CPT1A, apo CIII, and HMGCS genes, to verify that the human hepatocytes used are responsive to PPARα agonists (Fig. 9). Apo CIII expression levels did not change distinctively in rat or human primary hepatocytes treated with Wy 14,643 independent of the expression level of human or rat PPARα (data not shown). Although several reports showed that apo CIII expression was down-regulated by peroxisome proliferators in human liver and HepG2 cells (Clavey et al., 1999; Lawrence et al., 2001; Staels et al., 1995), other reports failed to show a PPARα-mediated regulation of apo CIII expression in human HepG2 cells (Hsu et al., 2001). This apparent discrepancies have been attributed to the culture conditions of hepatocytes, in particular the differences of cell culture media and characteristics of the supplemented serum (Clavey et al., 1999). Nevertheless, Wy 14,643 strongly induced CPT1A and HMGCS in both rat and human primary hepatocytes in the presence and absence of transfected human or rat PPARα, supporting that human PPARα is functional in the human hepatocytes used (Fig. 9).

FIG. 6. Quantification of ACOX mRNA in HepG2 and primary human hepatocytes. (A) HepG2 cells and (B) primary human hepatocytes were treated with 100 μM Wy 14,643 (solid columns) or ciprofibrate (striped columns) for 2, 24, or 72 h as indicated. Total RNA was analyzed for ACOX expression by TaqMan® analysis. Data are expressed as percentage of 18S rRNA expression. Shown are mean values with standard deviation of three independent experiments. Fold (x) inductions compared to vehicle control (open columns) are indicated on top of or next to each column. Significant differences of treated samples compared to vehicle control were determined using ANOVA/Dunnett’s test (n = 3, p < 0.05) and are indicated by an asterisk.

FIG. 7. Quantification of ACOX and PPARα mRNA in the absence or presence of transfected human or rat PPARα in FAO and HepG2 cells. (A) FAO or (B) HepG2 cells were transiently transfected with empty mammalian expression vector pSG5 (striped columns) or PPARα expression vector pSG5-hPPARα; solid columns) or rat PPARα (pSG5-rPPARα; open columns) for 6 h as described in the Materials and Methods section. After transfection, cells were treated with 100 μM Wy 14,643 (+) or with 0.5% (v/v) vehicle DMSO (−) for 24 h as indicated. Total RNA was analyzed for rat or human ACOX, human and rat PPARα expression by TaqMan® analysis. Data are expressed as percentage of 18S rRNA expression. Shown are mean values with standard deviation of three independent experiments. Fold (x) inductions of ACOX expression compared to vehicle control are indicated on top of each column.
DISCUSSION

It is well established that peroxisome proliferators exert distinct effects in rodents in contrast to humans. A number of studies have been published that analyzed various aspects of this species-specificity (for reviews see, e.g., Cattley et al., 1998; Vanden Heuvel, 1999), and we aimed to provide a comprehensive analysis of the involvement of PPARα, PPARG, and in particular the cellular context in the species-specificity of PPARα agonists.

To this end, we first analyzed induction of the marker enzymes ACOX and CAT as well as catalase in rat and human hepatocytes. Confirming earlier reports (Duclos et al., 1997; Perrone et al., 1998; Stangl et al., 1995), fibrates induced strongly ACOX and CAT but to a lesser extent catalase in rat hepatocytes whereas human HepG2 cells were refractory. To investigate whether the observed distinct enzyme induction is due to differences in the PPRE sequence of the ACOX gene (Lambe et al., 1999), the PPARGα (Lawrence et al., 2001), or the cellular environment of rat vs. human, we analyzed the activity of either rat or human PPARGα on a rat or human ACOX-derived PPRE (Tugwood et al., 1992; Varanasi et al., 1996, 1998) in rat and human hepatocytes. These experiments confirmed previously published reports (Hasmall et al., 2000; Lambe et al., 1999) that the human PPARGα does not activate a human PPRE derived from the ACOX promoter in either rat FAO or human HepG2 cells when treated with PPARGα agonists. Human PPARGα was not able to significantly activate the
rat ACOX PPRE or the full-length ACOX promoter in human hepatocytes; an observation that was also evident for rat PPARα and may reflect the nonresponsiveness of human hepatocytes as discussed in more detail later. In addition, human PPARα did not activate the rat ACOX PPRE in rat FAO or primary hepatocytes. In contrast, others showed that human PPARα was active on a rat ACOX promoter in murine cells (Hasmali et al., 2000; Sher et al., 1993). We also observed induction of the rat ACOX promoter in rat hepatocytes in the presence of human PPARα, although this activity could not be discriminated from endogenous rat PPARα. Taken together, overexpressed human PPARα exerted some activity on the rat ACOX promoter; however, in our hands, this activity was lower than that of rat PPARα at comparable mRNA expression levels. Lambe and colleagues (1999) compared the identical human and rat PPRE sequences used in this study in transactivation assays using the mouse PPARα. They showed that the human PPRE conferred no activity to mouse PPARα whereas the rat PPRE was responsive to peroxisome proliferators (Lambe et al., 1999). We confirmed that rat PPARα did not activate through the human ACOX PPRE but distinctively induced the rat ACOX PPRE and the full-length rat ACOX promoter. The published results and our study showed that both human PPARα and human PPRE sequence limit the activation of peroxisome proliferation related gene transactivation in hepatocytes.

Next we investigated whether the expression level of human PPARα could account for the observed lack of peroxisome proliferation related effects in human hepatocytes. Human PPARα is expressed at low levels in liver compared to rodents (Braissant et al., 1996; Lemberger et al., 1996; Palmer et al., 1998; Tugwood et al., 1996) and we confirmed these different expression levels in the cell lines used for this study. Nevertheless, HepG2 and primary human hepatocytes had significant levels of PPARα reaching up to 25% mRNA expression level compared to the rat FAO cells. In addition, overexpression of human PPARα up to a level comparable to that observed for rat PPARα in primary rat hepatocytes did not increase the induction of ACOX in human hepatocytes. Our observation was confirmed by other studies that showed that overexpression of human PPARα in HepG2 cells did not increase the responsiveness to peroxisome proliferators with regard to ACOX mRNA expression and enzyme activity (Hsu et al., 2001; Lawrence et al., 2001). In contrast to these studies and our data are results obtained in hepatocytes of guinea pigs, a species that is not responsive to peroxisome proliferators (Macdonald et al., 1999). In guinea pig hepatocytes, transfection of human or guinea pig PPARα increased nafenopin-induced peroxisome proliferation related effects (Macdonald et al., 1999). It is therefore likely that regulation of the peroxisomal marker enzyme palmitoyl-CoA oxidase may be differently regulated in guinea pig hepatocytes compared to human hepatocytes. Taken together, the lack of human PPARα activity in human hepatocytes is unlikely to be due to the low level of human PPARα expression. Palmer and colleagues (1998) showed that splice variants of human PPARα exist that lack activity and are dominant-negative (Gervois et al., 1999). Our analysis of mRNA PPARα expression was done with a TaqMan® probe that is located upstream of the spliced exon 6 of the inactive PPARα variant (Palmer et al., 1998). The observed amounts of human PPARα mRNA in human hepatocytes correspond therefore to the total amount of wild-type and inactive human PPARα variant. This inactive PPARα splice variant could therefore contribute to the lack of peroxisome proliferation related effects observed in human hepatocytes.

We then scrutinized whether the above-described factors are sufficient to determine the species-specificity of peroxisome proliferation related effects. Hsu and colleagues showed that human hepatocytes, although refractory to peroxisome proliferation related effects, are susceptible to PPARα-mediated expression of CPT1A and HMGCS, genes that are involved in lipid metabolism in human liver (Hsu et al., 2001). Here, we demonstrated that the primary human hepatocytes used maintained their PPARα responsiveness with regard to CPT1A and HMGCS expression. In addition, Lawrence et al. showed that overexpressed human or murine PPARα increased expression of HMGCS but not ACOX in HepG2 cells (Lawrence et al., 2001). Consistent with this report, overexpression of rat PPARα did not increase ACOX induction in human hepatocytes, which is likely due to the lack of responsiveness of the PPRE in the human ACOX promoter (Lambe et al., 1999). Intriguingly, we could not observe a distinct induction of rat or human ACOX PPRE or the full-length rat ACOX promoter in human hepatocytes when either human or rat PPARα was overexpressed to mRNA levels comparable to that observed in primary rat hepatocytes. Macdonald et al. showed that nafenopin induced activity of human and murine PPARα on a rat ACOX PPRE in guinea pig hepatocytes (Macdonald et al., 1999). However, others showed that Wy 14,643 induced weakly—about 2-fold and less compared to the vehicle control—the rat ACOX PPRE in HepG2 cells in the presence of overexpressed human PPARα (Hsu et al., 2001) consistent with our results. Hsu et al. demonstrated that a murine PPARα mutant with an increased sensitivity for ligand-induced activation retained responsiveness to peroxisome proliferators in HepG2 cells (Hsu et al., 2001). However, wild-type murine as well as human PPARα displayed no distinct responsiveness to peroxisome proliferators on a rat ACOX PPRE in HepG2 cells (Palmer et al., 1998) similar to our results. Since the rat PPARα expression plasmid used in our study yielded similar transfection efficiencies and also comparably high mRNA expression levels in both rat and human hepatocytes and hepatoma cell lines, we conclude that human hepatocytes impede PPARα-mediated induction of both rat and human ACOX reporter gene activity. However, depending on the properties of PPARα, the target gene promoter or the potency of the PPARα ligand, this unfavorable environment for peroxisome proliferation in human hepatocytes may be overcome.
In conclusion, our results confirmed that the differences in promoter sequences of PPARα target genes and the PPARα contribute to the species-specificity of peroxisome proliferators in hepatocytes. However, these factors are not sufficient to determine the species-specificity of peroxisome proliferation, since human hepatocytes harnessed a marked induction of peroxisome proliferation marker genes and PPARα activity. It is therefore conceivable that hepatocytes may lack or overexpress co-regulators in a species-specific manner that might facilitate or inhibit PPARα-mediated gene expression. Identification of these specific factors, which consequently may also be involved in the tumorigenic effects of PPARα agonists in the rodent liver, would further enhance our understanding of the molecular mechanisms of the species-specificity of peroxisome proliferators.

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