Differential Gene Regulation in Human Versus Rodent Hepatocytes by Peroxisome Proliferator-activated Receptor (PPAR) α

PPARα FAILS TO INDUCE PEROXISOME PROLIFERATION-ASSOCIATED GENES IN HUMAN CELLS INDEPENDENTLY OF THE LEVEL OF RECEPTOR EXPRESSION

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We compared the ability of rat and human hepatocytes to respond to fenofibric acid and a novel potent phenylacetic acid peroxisome proliferator-activated receptor (PPAR) α agonist (compound 1). Fatty acyl-CoA oxidase (FACO) activity and mRNA were increased after treatment with either fenofibric acid or compound 1 in rat hepatocytes. In addition, apolipoprotein CII mRNA was decreased by both fenofibric acid and compound 1 in rat hepatocytes. Both agonists decreased apolipoprotein CII mRNA in human hepatocytes; however, very little change in FACO activity or mRNA was observed. Furthermore, other peroxisome proliferation (PP)-associated genes including peroxisomal 3-oxoacyl-CoA thiolase (THIO), peroxisomal enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (HD), peroxisomal membrane protein-70 (PMP-70) were not regulated by PPARα agonists in human hepatocytes. Moreover, other genes that are regulated by PPARα ligands in human hepatocytes such as mitochondrial HMG-CoA synthase and carnitine palmitoyl transferase-1 (CPT-1) were also regulated in HepG2 cells by PPARα agonists. Several stably transfected HepG2 cell lines were established that overexpressed human PPARα to levels between 6- and 26-fold over normal human hepatocytes. These PPARα-overexpressing cells had higher basal mRNA levels of mitochondrial HMG-CoA synthase and CPT-1; however, basal FACO mRNA levels and other PP-associated genes including THIO, HD, or PMP-70 mRNA were not substantially affected. In addition, FACO, THIO, HD, and PMP-70 mRNA levels did not increase in response to PPARα agonist treatment in the PPARα-overexpressing cells, although mitochondrial HMG-CoA synthase and CPT-1 mRNAs were both induced. These results suggest that other factors besides PPARα levels determine the species-specific response of human and rat hepatocytes to the induction of PP.

Chemicals that elicit the phenomenon of peroxisome prolif-eration in rodents are associated with hepatocarcinogenesis in long-term studies (reviewed in Ref. 1). The tumorigenic response seems related to both the oxidative stress and increased cell proliferation observed after treatment with these chemicals. It has been demonstrated that non-rodent species are refractory to the induction of peroxisome proliferation (2–4); however, humans are not totally unresponsive to treatment with PPARα agonists. For example, the expression of apo CIII, a lipoprotein involved in triglyceride transport, is suppressed by PPARα agonists in rodents and humans and is involved in the hypotriglyceridemic effects of PPARα agonists observed clinically (5). In addition, mitochondrial HMG-CoA synthase, a gene involved in regulating ketogenesis, is responsive to PPARα agonist treatment independently of the ability of the species to respond by inducing peroxisome proliferation (6). Thus, species differences in response to peroxisome proliferation induction appear distinct from PPARα activation. There are several potential explanations for the species-specific differences in response to the induction of peroxisome proliferation. One hypothesis suggests that the difference between rodents and non-rodents in their susceptibility to peroxisome proliferation is related to the difference in relative expression of PPARα between the species (7–10). For example, human liver seems to express PPARα at levels that are approximately an order of magnitude lower than rat liver (11). It is possible, if the amount of the PPARα is limiting, that only a subset of genes may be induced upon exposure to ligand. Another hypothesis is that the response element is defective in non-rodent species (12, 13) and that the receptor cannot bind to or regulate the genes because they do not have functional PPREs within their promoters. Indeed, when several human genomic samples were analyzed, sequence analysis found that their fatty acyl-CoA oxidase promoter contained disrupted PPRE sequences.

In these studies, we compared the response of rat and human hepatocytes to the effects of fenofibric acid and a novel potent phenylacetic acid PPARα agonist (compound 1). In addition, we developed several HepG2 cell lines with different levels of

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† The abbreviations used are: PPARα, peroxisome proliferator-activated receptor α; apo CIII, apolipoprotein CIII; PPRE, peroxisome proliferator response element; hPPARα, human PPARα; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; GST, glutathione S-transferase; LBD, ligand binding domain; CBP, CREB-binding protein; PCR, polymerase chain reaction; RT, reverse transcriptase; FACO, fatty acyl-CoA oxidase; CPT-1, carnitine palmitoyl transferase-1; THIO, peroxisomal 3-oxoacyl-CoA thiolase; HD, peroxisomal enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase; CYP4A, cytochrome P450 4A; PMP-70, peroxisomal membrane protein-70; mtHMGs, mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase.
human PPARα overexpression and determined the effect of overexpression on basal and ligand-stimulated expression of several genes known to be responsive in both rat and human hepatocytes as well as peroxisome proliferation-associated genes that are only responsive in rat hepatocytes.

**EXPERIMENTAL PROCEDURES**

**Primary Hepatocytes**—Rat hepatocytes were isolated by a standard two-stage collagenase perfusion (14), plated in Williams E medium containing 10% fetal bovine serum, 1 μM dexamethasone, 1 μM insulin, 20 μg/ml gentamicin, 292 mg/ml L-glutamine, and 50 mM HEPES on rat-tail collagen, and allowed to attach overnight at 37 °C. Approximately 24 h after isolation, fresh medium with all additions except fetal bovine serum and containing the appropriate concentration of fenofibric acid or compound 1 (3-chloro-4-(3-(3-phenyl-7-propyl-1-benzofuran-6-yl)oxy)propyl)thio)phenyl)acetic acid, Merck Research Laboratories, Rathway, NJ) was added to the cells as a 100× stock in Me2SO. The culture medium was replaced at 24 h and after 48 h of treatment, the cells were rinsed with 100 mM NaPO4, pH 7.4, and harvested by scraping. The cells were disrupted by sonication and the resulting homogenate were assayed for protein concentration by the Lowry procedure (15) and frozen at −70 °C. Human hepatocytes were obtained commercially from Clonetics Corp. (San Diego, CA) as cultures already attached to collagen in proprietary medium. They arrived in collagen solution and were recovered overnight at 37 °C prior to use. Subsequently, the human hepatocytes were treated and processed as indicated above with the exception that the Clonetics proprietary medium was used at medium changes and during treatment.

**Homogeneous Time-resolved Fluorescence-based Nuclear Receptor Association Assay**—A homogeneous time-resolved fluorescence assay-based nuclear receptor-activator assay was used to examine the interaction of hPPARα with compound 1. A complete description of this assay has been published elsewhere (16). Briefly, 198 μl of reaction mixture (100 mM HEPES, 125 mM KF, 0.125% (w/v) CHAPS, 0.05% dry milk, 5 mM GST-hPPARα-LBD, 2 mM anti-GST-Eu(K), 10 mM biotin-CBP-(1–453), and 20 mM SAXL665) were added to each well followed by the addition of 2 μl of Me2SO or compound 1 (in Me2SO) in appropriate wells. The plates were mixed by hand and covered with TopSeal. The plates were incubated overnight at 4 °C followed by fluorescence measurement on a Discovery instrument (Packard). The data were reported as a ratio of the fluorescence at 665 nm (X665, A counts) to the fluorescence at 620 nm (Eu(K), B counts) multiplied by 10,000 (to give a white light signal). The GST-hPPARα-LBD was constructed by PCR the cloning of the hPPARα sequence encoding the ligand binding domain (from amino acid 167 to 468) into a GST vector. The GST-hPPARα-LBD fusion protein was purified as described (16).

**PPARα-GAL4 Chimeric Receptor Transactivation Assay**—Transactivation by human or murine PPARα was determined in transiently cotransfected in COS-1 cells as described (17) using pSG5-hPPARα-GAL4 or pSG5-mPPARα-GAL4, and both pUAS/GST-tk-luciferase and pCMV-lacz were then incubated with the indicated concentrations of compound 1 for 48 h.

**HepG2 Cells and Stably Transfected HepG2 Cells Overexpressing hPPARα**—HepG2 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. The hPPARα expression construct, pIREsneo-hPPARα, was constructed by PCR subcloning of the sequence encoding full-length hPPARα into pIREsneo (CLONTECH). The hPPARα coding sequence was resequenced to confirm the absence of PCR error. HepG2 cells were transfected with pIREsneo-hPPARα using a standard LipofectAMINE method (Life Technologies, Inc.). Stably transfected cell lines were selected in the presence of 0.5 mg/ml G418 for 4 weeks with the culture medium changed twice a week. Individual G418-resistant colonies were then trypsinized and cultured for 5 days followed by immunoblot and RNA analysis by the 5′-nuclease RT-PCR assay to verify hPPARα expression. The cell lysates were obtained by the direct addition of SDS sample buffer without dithiothreitol. The protein concentration was determined using the BCA method (Pierce). Western blots were performed as described using an anti-PPARα antibody (18).

**Fatty Acyl-CoA Oxidase Activity**—Fatty acyl-CoA oxidase activity was determined using the evolution of H2O2 as described by Poosch and Yamazaki (19) using lauryl-CoA as the substrate and 1 mM hydroxyphenylacetic acid as the indicator. Incubations were carried out at 37 °C for 10 min for the rat homogenates and 15 min for the human homogenates and stopped with 2 mM KCN in carbonate buffer. All samples were assayed in duplicate with a corresponding blank (lacking lauryl-CoA) subtracted. The results were converted to nanomoles of product by comparison to an H2O2 standard curve and normalized to milligrams of protein.

**Northern Analysis**—Total RNA was isolated from cells with 1 ml of Triazol reagent (Life Technologies, Inc.) following manufacturer instructions. RNA was quantified by spectrophotometry at 280 nm. Rat apo CIII mRNA and human apo CIII mRNA were treated and processed as indicated above with the exception that isolated rat or human total RNA using primers derived from published sequences (20, 21). Purified PCR-generated DNA fragments of rat and human apo CIII and an 18S rRNA DNA template were labeled with a psoralen-biotin kit. Northern analysis was performed using a Tris borate EDTA-urea polyacrylamide gel electrophoresis gel system. RNA was then electroblotted to a positively charged nylon membrane and probed with the labeled apo CIII probe overnight and washed, and the biotinylated probe was detected with a streptavidin-alkaline phosphatase conjugate and a chemiluminescent substrate. The chemiluminescent signal was detected by exposure to x-ray film and quantified by densitometry. Probes were stripped, and the filter was reprobed with biotinylated 18S rRNA probe, detected by chemiluminescence, and quantified as above. The data are expressed as the ratio of apo CIII to 18S rRNA.

**5′-Nuclease RT-PCR Assay**—Fatty acyl-CoA oxidase (FACO), carnitine palmitoyltransferase-1 (CPT-1), peroxisomal 3-oxoacyl-CoA thiolase (THIO), peroxisomal enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (HD), cytochrome P450 4A (CYP4A), peroxisomal membrane protein-70 (PMP-70), and mitochondrial HMG-CoA synthase (HMG-CoA synthase) were quantified using real-time RT-PCR. cDNA synthesis was performed in 25 μl with 0.1 μg total RNA with the TaqMAN® kit (PerkinElmer Life Sciences) with the following conditions: 25 °C for 10 min, 48 °C for 30 min, and 95 °C for 5 min. After reverse transcription, a 3-μl aliquot was transferred into a 25-μl TaqMAN® amplification reaction (TaqMAN® PE 2× PCR Mastermix diluted to a 1× final concentration) containing primer/probes for 18S rRNA (OE-tagged: 6-carboxy-4,5-dichloro-2′,7′-dimethyfluorescein) and either rat or human Faco, CPT-1, THIO, HD, CYP4A, PMP-70, or HMG-CoA synthase (6-FAM tagged: 6-carboxy-4,5-dichloro-2′,7′-dimethyfluorescein) with the following conditions: 50 °C for 2 min, 95 °C for 10 min and amplified at 95 °C for 15 s, and 60 °C for 1 min for 40 cycles. The specific sequences of the primers and probes are listed in Table I.

**RESULTS**

**PPARα Is Active in Both Rat and Human Hepatocytes But Regulates FACO Activity Only in Rat Hepatocytes**—After treatment with fenofibric acid for 48 h, a dose-dependent increase in FACO activity was observed in rat hepatocytes (Fig. 1). At 100 μM, fenofibric acid produced a 6-fold increase in FACO activity. No increase in FACO activity was observed in human hepatocytes. In parallel culture dishes, fenofibric acid produced a dose-dependent suppression of apo CIII mRNA levels in both rat and human at the doses that corresponded to those that induced FACO activity in rat hepatocytes. A novel highly potent phenylacetic acid PPARα agonist (compound 1) was used to assess the effects of a more potent PPARα agonist in this system. This compound has high potency on the human PPARα receptor but is a weak agonist on the murine receptor. Based on the ability of PPARα to recruit the CBP coactivator in vitro, the estimated EC50 for compound 1 on human PPARα is 16 nM and is 10 μM on the murine receptor (Fig. 2A). Concentrations greater than 10 μM could not be tested in the homogeneous time-resolved fluorescence-based nuclear receptor association assay because of the quenching effect of the compound. These values corresponded well with results using chimeric human or murine PPARα-GAL4 receptor transactivation reporter assays (Fig. 2B). When compound 1 was assessed in rat hepatocytes, FACO activity was induced slightly at doses above 1 μM, corresponding to the weak potency on rodent PPARα (Fig. 3). Doses higher than 10 μM of compound 1 were cytotoxic to the rat and human hepatocytes. Compound 1 also suppressed apo CIII mRNA at doses above 1 μM. Only a very modest effect of compound 1 was observed in human hepatocytes on FACO activity (~2-fold) at doses up to 10 μM, however, apo CIII mRNA was suppressed at doses as low as 10 nM.
apo CIII corresponded with its potency on the human PPAR\(\alpha\)/H9251.

PPAR\(\alpha\)/H9251 Agonists Do Not Regulate Peroxisome Proliferation-associated Genes in Human Hepatocytes or HepG2 Cells—The effects of fenofibric acid and compound 1 were assessed on genes potentially regulated by PPAR\(\alpha\)/H9251 in rat and human hepatocytes. Messenger RNA for the peroxisome proliferation-associated genes including FACO, THIO, HD, CYP4A1, and PMP-70 were induced by fenofibric acid and compound 1 in rat hepatocytes (Fig. 4A). In rat hepatocytes, fenofibric acid was more efficacious than compound 1 probably because of the ability to test higher doses of fenofibric acid in rat hepatocytes without inducing frank cytotoxicity. Because of the decreased potency and limited concentration of compound 1 that could be tested, a more modest increase was observed. In human hepatocytes, no increase in the peroxisome proliferation-associated genes was observed (Fig. 4B). A small increase in CYP4A11 mRNA was observed in human hepatocytes with compound 1; however, the magnitude was more than 25-fold less than that observed in rat hepatocytes. Two genes were responsive to fenofibric acid and compound 1 in rat hepatocytes, CPT-1 and mtHMGS. When the same set of genes was examined in HepG2 cells, no response to either fenofibric acid or compound 1 was observed for the mRNA for the peroxisome proliferation-associated genes FACO, THIO, HD, FACO, or PMP-70 (Fig. 4C). Similar to what was observed in human hepatocytes, CPT-1 and mtHMGS both were induced after treatment. Inter-

| Table I | Primer/Probe sequences |
|---------|------------------------|
| Gene    | Species | Primer 1 | Primer 2 | Probe |
| Peroxisomal fatty acyl-CoA oxidase | Rat | GTGCCAATGAGCTGTAACAGCCG | CATCCGAAATATGAG | TCCAGACGGCTAGGTTCCTGGTTCAAATAGGACACCATGCCAAAGTTATGATCAGGTGCACTCAGGAAAGTTGG |
| Peroxisomal 3-oxoacyl-CoA thiolase | Rat | GCCACACCAGCGCGAAGCTGAT | CTGAAGCAATGGGCTGTTGAGCAG | CAGAAGGCAGCAAGAGCCTGGACCGTGGTGGTCACAGAGAGCAAGGGCTGTTTCCAAGCTGAGATT |
| Peroxisomal enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase | Rat | AAGAGATTTGAGCAGTCTTCTACAT | TTGCCTGCACAGAGCTGACCTCAG | AAGATAATCACTTTCACCTTGGAGTTTGGTTTTGCCGAAGCTTGAGCATCCAGAGCGCATCAGAACGG |
| Peroxisomal membrane protein-70 | Rat | GTAGTTGGGTACCTGGTTGTCAGTCAGTGGGTGCTGTGAAGGTGTCCCCGTTCCTAGACCTGGCGCATC | GTAGTTGGGTACCTGGTTGTCAGTCAGTGGGTGCTGTGAAGGTGTCCCCGTTCCTAGACCTGGCGCATC |
| Cytochrome P450 4A1 | Rat | CCGGCTGCTGGATGGTGTCTGTCTCTTAACATCCGCTCCCACTGAGCGG | CCGGCTGCTGGATGGTGTCTGTCTCTTAACATCCGCTCCCACTGAGCGG |
| Carnitine palmitoyltransferase-1 | Human | CCGTCTTTTGGGATCCACGATTTGTGCTGGATGGTGTCTGTCTCTTAACATCCGCTCCCACTGAGCGG | CCGTCTTTTGGGATCCACGATTTGTGCTGGATGGTGTCTGTCTCTTAACATCCGCTCCCACTGAGCGG |
| Mitochondrial HMG-CoA synthase | Human | TGAGGGCATAGATACCACCAATGTGGCATAACGACCATCCCAATGCTGCCAACTGGATGGAGTCCAG | TGAGGGCATAGATACCACCAATGTGGCATAACGACCATCCCAATGCTGCCAACTGGATGGAGTCCAG |

![Graph](http://www.jbc.org/)

**Fig. 1.** Fenofibric acid induces FACO activity and suppresses apo CIII mRNA in rat hepatocytes but only suppresses apo CIII mRNA in human hepatocytes. Rat or human primary hepatocytes were cultured for 48 h in the presence or absence of various concentrations of fenofibric acid and then harvested for FACO activity determinations or apo CIII mRNA Northern analysis as described under “Experimental Procedures.” The apo CIII signal was corrected by reprobing with an 18S rRNA probe and dividing the apo CIII signal by the 18S rRNA signal. The data are expressed as nmol of H2O2/min/mg of protein for FACO activity or relative to untreated controls for apo CIII (mean ± S.E., n = 4). ○, rat hepatocytes; ●, human hepatocytes.
FIG. 2. Compound 1 is a potent human but weak murine PPARα agonist. A, the ability of compound 1 (the inset shows the structure) to promote human (●) or murine (○) PPARα and CBP interaction was analyzed using an homogeneous time-resolved fluorescence-based nuclear receptor assay in the presence of 10 nM SA/XL665, 10 nM biotin-CBP-1–453), 1 nM GST-PPARαLBD, and 2 nM anti-GST-EuK as described under “Experimental Procedures.” The 665 nm/620 nm ratio for each well was normalized by subtracting that obtained from the control well followed by calculation of the percentage maximum activation (using maximal ligand-induced fluorescence resonance energy transfer as 100%). The titration using human GST-PPARαLBD is shown. Each point represents the mean ± S.E. of three determinations. The experiment was repeated with similar results. B, transactivation by human (●) or murine (○) PPARα in COS-1 cells transiently cotransfected with pSG5-PPARα-GAL4 or pSG5-mPPARα-GAL4 chimeric constructs and both pUAS(5X)-tk-luciferase and pCMV-lacZ then incubated with the indicated concentrations of compound 1 for 48 h. The data are expressed as the mean ± S.E. of normalized luciferase activity (n = 3).

Certainly, mtHMGS and CPT-1 basal mRNA levels were much lower in the HepG2 cells than in human hepatocytes. Basal mRNA levels of FACO, THIO, and HD were similar to those in human hepatocytes, whereas basal mRNA levels of PMP-70 were 4–5-fold higher than those in human hepatocytes. There was no detectable CYP4A11 mRNA in HepG2 cells.

Overexpression of hPPARα Does Not Sensitize Human Cells to Induction of Peroxisome Proliferation-associated Genes—Several stable HepG2 cell lines were created that overexpress human PPARα. The cell lines expressed from 6- to 26-fold higher levels of human PPARα mRNA than in primary human hepatocytes (Fig. 5). When three of these cell lines were assessed by Western analysis, they demonstrated markedly increased expression of PPARα protein. Some cell lines had higher protein levels than would be expected from their mRNA analysis (compare cell line J35 to J14). Basal levels of the selected mRNAs mentioned above were measured via the 5′-nucleotide RT-PCR assay. Increasing levels of PPARα overexpression in these cells increased the basal levels of mtHMGS and CPT-1 mRNA (Fig. 6). The effect on basal levels of mtHMGS mRNA appeared to be saturable; however, the basal regulation of CPT-1 mRNA was not. In contrast, the peroxisome proliferation-associated genes FACO, THIO, HD, and PMP-70 were not mRNA affected substantially by increasing the amount of PPARα in the cell. PMP-70 did increase modestly, but that may be because of the much higher basal levels in HepG2 cells compared with human hepatocytes. The regulation of the same set of genes was assessed in the J35 cell line (10-fold higher PPARα levels than human hepatocytes) after treatment with 100 nM compound 1 (Fig. 7). Similar to the results found in primary human hepatocytes, both mtHMGS and CPT-1 mRNA were strongly induced after treatment; however, mRNA for the peroxisome proliferation-associated genes FACO, THIO, HD, and PMP-70 were not affected substantially by compound 1 treatment (Fig. 7). PMP-70 mRNA levels were increased modestly by compound 1; however, the magnitude of this change was small (less than 2-fold) compared with the induction observed in rat hepatocytes (6–7-fold). Thus, no meaningful induction of peroxisome proliferator-associated genes was observed regardless of the amount of PPARα in the cell.

DISCUSSION

We compared rat and human cells to investigate mutual versus species-specific responses to PPARα agonists. Rat hepatocytes responded to PPARα agonist treatment by inducing the mRNA for several peroxisome proliferation-related genes including FACO, HD, THIO, CYP4A, and PMP-70 and had increased fatty acyl-CoA oxidase activity. In addition, PPARα agonist treatment suppressed apo CIII mRNA at the same
doses that induced FACO activity. Fibrates regulate apo CIII through a PPRE in the promoter of the apo CIII gene (5), and the regulation of apo CIII is absent in PPARα knockout mice.

Thus, suppression of apo CIII mRNA can be considered a measure of PPARα activation in vivo. Consistent with their in vitro pharmacologic profile on murine PPARα/H9251, the potency of fenofibric acid and compound 1 were similar at suppressing apo CIII mRNA levels in rat hepatocytes. In addition, compound 1 produced similar effects as other PPARα agonists in rat hepatocytes on FACO activity.

Both compound 1 and fenofibric acid suppressed apo CIII mRNA levels in human hepatocytes; however, compound 1 was found to have at least a 1000-fold greater potency than fenofibric acid in these cells. This difference in potency correlated with the ability of compound 1 to function as a very potent human PPARα agonist as assessed by its ability to transactivate a reporter construct or recruit CBP in the in vitro interaction assay, which is a more direct measure of PPARα affinity.

Mukherjee et al. (22) have observed that WY14,643 was more potent at activating the rat PPARα than the human PPARα. In FIG. 4, Fenofibric acid and compound 1 induce mitochondrial HMG-CoA synthase and CPT-1 in human and rat hepatocytes and HepG2 cells, but peroxisome proliferator-associated genes are induced only in rat hepatocytes. Rat hepatocytes (A), human hepatocytes (B), or HepG2 cells (C) were cultured for 48 h in the absence (open bars) or presence of 100 μg Fenofibric acid (solid bars) or 100 nM compound 1 (hatched bars) and harvested, and total RNA was isolated as described under “Experimental Procedures.” The specific mRNAs were quantified by the 5'-nuclease RT-PCR assay and expressed relative to untreated cells (rat and human hepatocytes) or relative to untreated human hepatocytes (mean ± S.D., n = 2–4).

Fig. 5. Stable hPPARα-expressing cell lines express increased amounts of hPPARα. Total protein extracts (40 μg) from HepG2 cells or stable HepG2 cells that overexpress hPPARα were analyzed by SDS-polyacrylamide gel electrophoresis (4–20%) and Western blotting with an antibody against PPARα as described under “Experimental Procedures.” The arrow indicates hPPARα protein. The B56 cell line was not available for Western analysis. Human PPARα mRNA levels were determined from total RNA from each HepG2 cell line by the 5'-nuclease RT-PCR assay, and the amount of hPPARα mRNA was expressed relative to untreated human hepatocytes.

FIG. 6. Basal mRNA levels of mitochondrial HMG-CoA synthase and CPT-1 are regulated in HepG2 cells expressing increased hPPARα levels, but peroxisome proliferator-associated genes are not. Stable hPPARα-overexpressing cells were harvested, and total RNA was isolated as described under “Experimental Procedures.” The specific mRNAs were quantified by the 5'-nuclease RT-PCR assay and expressed relative to untreated human hepatocytes. Open bars, parent HepG2; solid bars, J38 line with 6-fold higher PPARα levels than human hepatocytes; right-hatched bars, J35 line with 10-fold higher PPARα levels than human hepatocytes; left-hatched bars, J14 line with 16-fold higher PPARα levels than human hepatocytes; cross-hatched bars, B56 line with 26-fold higher PPARα levels than human hepatocytes.

(data not shown). Thus, suppression of apo CIII mRNA can be considered a measure of PPARα activation in vivo. Consistent with their in vitro pharmacologic profile on murine PPARα, the potency of fenofibric acid and compound 1 were similar at suppressing apo CIII mRNA levels in rat hepatocytes. In addition, compound 1 produced similar effects as other PPARα agonists in rat hepatocytes on FACO activity.

Both compound 1 and fenofibric acid suppressed apo CIII mRNA levels in human hepatocytes; however, compound 1 was found to have at least a 1000-fold greater potency than fenofibric acid in these cells. This difference in potency correlated with the ability of compound 1 to function as a very potent human PPARα agonist as assessed by its ability to transactivate a reporter construct or recruit CBP in the in vitro interaction assay, which is a more direct measure of PPARα affinity.
addition, they observed that ETYA was more potent on the human PPARα than on the rat PPARα. Our results are consistent with these findings and suggest that human and rodent PPARαs can be distinguished pharmacologically. There are two amino acids that differ in the ligand-binding domain of PPARα between rat and human. These differences in amino acid sequence may explain the differences in the potency of the various ligands for each of the species receptors.

In contrast to rat hepatocytes, the doses of fenofibric acid or compound 1 that effectively suppressed apo CIII mRNA levels were not associated with FACO activity increases in human cells, even though apo CIII mRNA levels were suppressed at doses as low as 10 nM compound 1. Thus, when a highly potent human PPARα ligand is incubated with human cells, even at doses 1000 times higher than required to fully activate the human PPARα (as measured by apo CIII mRNA suppression), no substantial induction of the peroxisome proliferation-associated genes was observed. These data dispel the concern that the previously assessed ligands in human hepatocytes were not potent enough to fully activate the human PPARα receptor. In addition, our data demonstrate that the maximal PPARα activation potential of both fenofibric acid and compound 1 were similar in rat and human hepatocytes because apo CIII mRNA was ultimately suppressed by ~80% in both species. Our data are also consistent with previous studies that have found that species other than rats and mice are resistant to the induction of peroxisome proliferation in vitro (2, 5, 23, 24).

Our data also demonstrate that HepG2 cells responded to PPARα agonist treatment in a similar fashion to human hepatocytes by inducing CPT-1 and mtHMGS mRNAs. Previous reports have demonstrated that CPT-1 and mtHMGS are regulated by PPARα through PPReS in their respective promoters (6, 25, 26). None of the peroxisome proliferation-associated genes including FACO, HD, THIO, or PMP-70 were responsive to PPARα agonist treatment in HepG2 cells.

Several PPARα-overexpressing HepG2 cell lines were studied to assess the effects of PPARα expression levels on basal and ligand-stimulated regulation of CPT-1 and mtHMGS and the peroxisome proliferation-associated genes. We found that HepG2 cells expressed similar levels of FACO, THIO, and HD mRNA as human hepatocytes. However, HepG2 cells have lower peroxisomal β-oxidation rates than human hepatocytes as well as lower protein levels of these genes (27). HepG2 cells cultured under our conditions had markedly higher PMP-70 mRNA levels than human hepatocytes. Thus, the regulation of the peroxisomal β-oxidation proteins may occur, in part, independently of PPARα and through a post-transcriptional mechanism. Interestingly, we found that genes regulated in parental HepG2 cells by PPARα ligands (CPT-1 and mtHMGS) were also induced by the stable overexpression of PPARα. In contrast, genes that were not responsive to PPARα ligands in human hepatocytes or HepG2 cells were likewise unaffected by increasing PPARα content in the HepG2 cells. Furthermore, treatment with compound 1 induced both the regulated genes (CPT-1 and mtHMGS) even further in the PPARα-overexpressing HepG2 cells but had no effect on the peroxisome proliferation-associated genes. Thus, increasing PPARα expression levels to those found in rat hepatocytes failed to render human cells responsive to the induction of classical markers of peroxisome proliferation. It has been observed that HepG2 cells in long-term culture can display features of a more differentiated state (28). During this process PPARα mRNA expression levels increased up to 4-fold; however, basal levels of FACO, HD, THIO, and PMP-70 mRNA remained unchanged, consistent with these studies using the stably transfected HepG2 cells lines.

One question that remains is whether genes implicated in mediating cell proliferation are also affected by PPARα overexpression. Our study did not include such measurements because of the transformed phenotype of the HepG2 cells. Furthermore, unlike the well characterized gene pathways that modulate peroxisomal or mitochondrial fatty acid oxidation, PPARα-regulated genes that may contribute to observed increases in cell proliferation in rodent hepatocytes are not well defined.

There are numerous hypotheses that attempt to address the mechanism of species differences in the responsiveness to the induction of peroxisome proliferation between rodents and humans (7, 29–31). This issue is central for understanding potential human risk associated with the use of fibrates and the development of newer PPARα agonists for dyslipidemia, atherosclerosis, or other indications. Our understanding of the mechanism of action of peroxisome proliferators has been improved through the identification of PPARα as the central regulator of the pleiotropic responses of these agents in rodents (32, 33). It has been hypothesized that the difference in responsiveness of rodents and non-rodents to peroxisome proliferators depends on the amount of PPARα in each species. Humans express ~10 times less PPARα in the liver than rats (11, 22, 34). Our studies have demonstrated clearly that increasing the amount of human PPARα in a human liver cell line does not result in concomitant increased sensitivity to the induction of peroxisome proliferation. Genes that were previously regulated in human cells by PPARα ligands continued to be responsive to PPARα ligands after overexpression. Neither basal nor ligand-stimulated regulation of the peroxisome proliferation-associated genes was conferred by overexpressing PPARα. MacDonald et al. (35) have reported that the transfection of guinea pig hepatocytes with murine PPARα conferred responsivity to the induction of peroxisome proliferation. These studies found a 20-fold induction of peroxisomal β-oxidation in primary rat hepatocytes and a less than 3-fold induction in primary guinea pig hepatocytes. Basal levels of peroxisomal β-oxidation in guinea pig hepatocytes were modified modestly by PPARα transfection; however, ligand-stimulated peroxisomal β-oxidation...
tion was increased by only 3-fold, the same as in untransformed guinea pig hepatocytes. The variability in these studies was quite high (50% or more in many cases) and may be the result of variable transfection efficiencies between samples. It could be possible that PPARα modulated the expression of another transcription factor that interacts with the acyl-CoA oxidase promoter and modified basal peroxisomal β-oxidation activity. This would explain why the response to ligand remained unchanged. It is unknown whether the guinea pig acyl-CoA oxidase promoter contains a functional PPRE. The sequence of the guinea pig acyl-CoA oxidase promoter will be required to resolve the role of PPARα in guinea pig responsivity to peroxisome proliferation.

Another hypothesis suggests that humans are less sensitive to peroxisome proliferation induction because human cells express a dominant negative form of PPARα derived from a truncated transcript (36). Our data suggest a different mode of regulation than through depletion of co-factors as suggested by Gervois et al. (36), because providing as little as 6-fold additional PPARα (based on mRNA levels) was sufficient to confer the ability to regulate the basal levels of the responsive genes CPT-1 and mHMGS. When more than 6-fold additional PPARα was present, basal CPT-1 and mHMGS mRNAs were induced by more than 20-fold. Moreover, treatment with ligand stimulated an additional 5-fold induction of mHMGS but not FACS mRNA or the other peroxisome proliferation-associated genes after PPARα overexpression. Thus, CPT-1 and mHMGS were regulated over 100-fold without changes in the expression of the peroxisome proliferation-associated genes. Further evidence that refutes the truncated-PPARα hypothesis arises from studies that have demonstrated that human cells transfected with a rat FACS/H9251 are responsive to PPARα agonist treatment by inducing the reporter gene construct (22). This regulation was present in either rat or human cell lines and with rat or human PPARα (22, 37). Thus, the human PPARα can regulate the rat PPRE in either mouse or human cells, suggesting that the cellular machinery in both human and rat cells is capable of regulating a promoter containing a functional PPRE.

An attractive hypothesis to explain the species differences in peroxisome proliferation induction suggests that a change in the sequence of the promoter of the acyl-CoA oxidase gene prevents the association of PPARα with the promoter of the peroxisome proliferation-associated genes (12, 37). Our data suggest that additional genes associated with peroxisome proliferation such as HD, THIO, and PMP-70 are likewise unresponsive, even after the overexpression of PPARα. This may be caused by similar sequence defects resulting in the loss of functional PPREs in the respective gene promoters. Alternatively, potential differences in one or more transacting factors (i.e., coactivators or corepressors) could be invoked to explain deviations between human and rodent cells that result in relative changes that affect a larger “cassette” of peroxisome proliferation-associated genes.

Our data and those reported previously lead us to hypothesize that the nonresponsive nature of humans and other species (besides rats and mice) to the induction of peroxisome proliferation is based on an evolutionarily controlled deviation intrinsic to cis-acting elements and/or trans-acting factors that are required for the induction of a cassette of genes that regulate fatty acid metabolism mainly involving peroxisomal fatty acid oxidation. Other genes involved in fatty acid and lipid metabolism continue to be controlled by PPARα in the liver of the peroxisome proliferation-resistant species, including humans, and provide a potential pharmacological benefit of PPARα agonist therapy currently used to treat hyperlipidemia. The risk of PPARα agonist-induced peroxisome proliferation and subsequent tumorigenesis seems unlikely given our new understanding of the role of PPARα and its spectrum of responsive genes in non-rodent species. However, further work is required to fully assess the differences in the promoter regions of the genes found to be unresponsive in human liver cells.

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